Brainstem Control Of Spinal Sensory Processing In The Rat

By

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ABSTRACT

Brainstem structures engage descending facilitatory and inhibitory neurones to potentiate or suppress the onward passage of sensory inputs from spinal loci to the brain. The final output for this bidirectional control is the rostral ventromedial medulla (RVM), which shapes sensory processing via relays between the spinal cord and brain, ultimately influencing pain perception via On and Off cells. I aimed to determine the predominant nature of this supraspinal control in the normal anaesthetised state and to see how descending influences may change with pathophysiology. By injecting a local anaesthetic into the RVM of normal rats and measuring the evoked responses of dorsal horn neurones to various stimuli, I demonstrated a dominant facilitation of spinal neuronal responses to high-threshold stimuli. Following nerve injury, reductions in spinal cord activity induced by intra-RVM lignocaine further encompassed responses to low-threshold stimuli, and the proportion of neurones influenced in this manner increased. The increase in descending facilitatory influences in the neuropathic state is suggestive of nerve injury induced plasticity. I additionally employed targeted ablation techniques using the neurotoxin saporin to show that mu-opioid receptor expressing cells in the RVM underlie spinal facilitation. This pathway involves spinal $5HT_3$ receptors, since typical responses to spinal ondansetron, a $5HT_3$ receptor antagonist, require the integrity of RVM MOR cells. Importantly, I showed that these neurones are critical for maintaining behavioural hypersensitivities following nerve injury, and are necessary for the full inhibitory efficacy of spinal pregabalin in the neuropathic state. These results define a pathway that could be pivotal for linking the sensory and affective components of pain. Understanding this connection, and how one component influences the other, may help explain the variable valence of suffering that is experienced by patients in response to the same nervous system injuries, and explain differences in treatment outcomes in an otherwise consistent population.

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ABBREVIATIONS

1°Ab	Primary Antibody
2°Ab	Secondary Antibody
ACT	Acquired Centralised Tinnitus
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
ANOVA	Analysis of Variance
AP	Action Potential(s)
BDNF	Brain Derived Neurotrophic Factor
Ca^{2+}	Calcium Ion
CCI	Chronic Constriction Injury
CCK	Cholecystokinin
m-CPBG	m-Chlorophenylbiguanide
cDNA	Complimentary Deoxyribonucleic Acid
CFA	Complete Freunds Adjuvant
cFOS	Protein Product of Gene cFos
CGRP	Calcitonin Gene Related Peptide
CINV	Chemotherapy-Induced Nausea and Vomiting
CNS	Central Nervous System
CRPS	Complex Regional Pain Syndrome
CTb	Cholera Toxin b
CVLM	Caudal Ventrolateral Medulla
CWMP	Chronic Widespread Muscle Pain
Derm-SAP	Dermorphin-Saporin
5,7-DHT	5,7-Dihydroxytryptamine
DLF	Dorsolateral Funiculus
DLPT	Dorso-Lateral Pontine Tegmentum
DMA	Dynamic Mechanical Allodynia
DN	Diabetic Neuropathy
DRG	Dorsal Root Ganglion
EAA	Excitatory Amino Acid
ERK	Extracellular Signal-Regulated Kinase
FDA	Food & Drug Administration

FITC	Fluroscein Iso-Thio-Cyanate
*f*MRI	Functional Magnetic Resonance Imaging
GABA	γ-Amino Butyric Acid
GAD	Glutamic Acid Decarboxylase
GBP	Gabapentin
GD	Gestational Day
GI	Gastrointestinal
GDNF	Growth Derived Neurotrophic Factor
GPCR	G-Protein-Coupled Receptor
HCN	Hyperpolarization-activated, Cyclic Nucleotide-modulated channels
HIV	Human Immunodeficiency Virus
5HT	5-Hydroxytryptamine, also known as Serotonin
IASP	International Association for the Study of Pain
IB4	Isolectin B4
i.cv	Intra-Cerebroventricular
Ir	Immunoreactivity
i.t	Intrathecal
IGluR	Ionotropic Glutamate Receptor
KOR	κ-Opioid Receptor
L4	Lumbar 4
L5	Lumbar 5
L6	Lumbar 6
LC	Locus Coeruleus
LTP	Long Term Potentiation
MAPK	Mitogen Activated Protein Kinase
2-me5HT	2-methyl 5-hydroxytryptamine
MGluR	Metabotropic Glutamate Receptor
MK-801	(5R, 10S)-(-)-5-Methyl-10, 11-dihydro-5H-dibenzo(a,d)cyclohepten-5, 10-imine maleate (Dizocilpine)
MOR	μ-Opioid Receptor
mRNA	Messenger Ribonucleic Acid
Na^+	Sodium Ion
NA	Noradrenaline
NC	Nucleus Cuneiformis

NGF	Nerve Growth Factor
NGS	Normal Goat Serum
NK	Neurokinin
NMDA	N-methyl-D-Aspartate
NNT	Number Needed to Treat
NRM	Nucleus Raphé Magnus
NRO	Nucleus Raphé Obscurus
NRP	Nucleus Raphé Pallidus
NS	Nociceptive Specific
NSAID	Non-Steroidal Anti-Inflammatory Drug
6-OHDA	6-Hydroxydopamine
PAG	Periaqueductal Gray
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGB	Pregabalin
PKA	Phosphokinase A
PKC	Phosphokinase C
PLC	Phospholipase C
PNS	Peripheral Nervous System
PO	Post-Operative
PSNL	Partial Sciatic Nerve Ligation
PSTH	Post-Stimulus Time Histogram
PWD	Paw Withdrawal
qPCR	Quantitative Polymerase Chain Reaction
QST	Quantitative Sensory Testing
RVM	Rostral Ventromedial Medulla
SAP	Saporin
SCI	Spinal Cord Injury
SEM	Standard Error of the Mean
SMA	Static Mechanical Allodynia
SMP	Sympathetically-Maintained Pain
SNI	Spared Nerve Injury
SNL	Spinal Nerve Ligation

SNRI	Serotonin and Noradrenaline Reuptake Inhibitor
SP	Substance P
SPA	Stimulation-Produced Analgesia
SP-SAP	Substance P-Saporin
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressants
TENS	Transcutaneous Electrical Nerve Stimulation
TNF-α	Tumour Necrosis Factor-alpha
TPH	Tryptophan Hydroxylase
Trk	Neurotrophin Receptor
TRP	Transient Receptor Potential
TRPV1	Vanilloid Receptor-1
TSA	Tyramine Specific Amplification
TTXr	Tetrodotoxin Resistant
TTXs	Tetrodotoxin Sensitive
VAS	Visual Analogue Scale
vF	von Frey
VGCC	Voltage Gated Calcium Channel
VGSC	Voltage Gated Sodium Channel
VLF	Ventro-Lateral Funiculus
VLM	Ventro-Lateral Medulla
VZV	Varicella Zoster Virus
WDR	Wide Dynamic Range
WT	Wild Type

CONTENTS

1. INTRODUCTION

1.1 THE PROBLEM OF PAIN

Pain is a unique, conscious experience that consists of sensory-discriminative, cognitive-evaluative and affective-emotional components. Recognition of the multi-dimensional nature of both the detection and perception of pain is central to the International Association for the Study of Pain (IASP)'s definition of this phenomenon as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'(Merskey, 1994)[1]. Stemming from this definition is the wide acknowledgement that there is no fixed, dependable relationship between the intensity of a potentially harmful, or 'noxious' stimulus and perceived pain intensity. Instead this relationship is governed, to different extents, by individual variation (including genetics), previous damage, and the condition of tissue and nerves. Furthermore, the relationship between noxious input and painful output is influenced by demographics (e.g. age and gender), the environmental context in which the stimulus is received, as well as the psychological and emotional states of the recipient. With so many contributory factors, it is at once easy to gage the problem of pain that challenges physicians and scientists alike, presenting millions of people worldwide with a largely unmet clinical need.

Transient and acute pain, which have a probable limited duration and identifiable temporal and causal relationship to nociception, injury or disease, serve as useful biological warning functions that protect the integrity and survival of an organism. However, when pain outlasts its usefulness, existing beyond the time it takes for injuries to heal (or possibly existing in the absence of any identifiable 'organic' cause), incurring secondary symptoms such as anxiety and depression, it becomes problematic and can greatly decrease function and quality of life. This progression towards pain deemed as 'chronic' means that pain becomes a syndrome in its own right rather than a by-product of some other process. It is in this respect that it should be attended to and treated. The most frequently used pain medication

[1] IASP is working on an addendum to this definition to avoid the possible suggestion that people who are not able to articulate their pain, infants or people with dementia for example, do not suffer pain.

remains the non-steroidal anti-inflammatory drugs (NSAIDs), followed closely by paracetamol and opioids (Pain in Europe Survey). However, neuropathic pain, a complex state that is associated with disease or injury within the peripheral or central nervous systems, often refracts standard analgesic therapy and may escalate in severity over time. Knowledge of the processes, both physiological and pharmacological, underlying chronic pain resulting from nerve injuries may lead to a shift from mediocre symptom control towards a more specific, mechanisms-based therapeutic approach. Thus, the aim and emphasis of this thesis is to further the understanding of pathways contributing to the generation and/or support of neuropathic pain.

1.2 NEUROPATHIC PAIN

1.2.1 CURRENT DEFINITION (INCLUDING CRITICISM) OF NEUROPATHIC PAIN

In 1994, the IASP taxonomy committee defined neuropathic pain as 'pain initiated or caused by a primary lesion or dysfunction of the nervous system' (Merskey & Bogduk, 1994). This definition has since come under scrutiny and has been criticised as being too encompassing, and as a consequence there are increasing demands for its revision. In particular it has been suggested that 'dysfunction' in the current definition should be replaced with 'disease'. This is because some dysfunctions of the nervous system such as allodynia (pain due to a stimulus that does not usually provoke pain) and hyperalgesia (an increased response to an already painful stimulus) are not forms of neuropathic pain, nor are they exclusive symptoms of neuropathic pain; these phenomena may also result from inflammatory pain as a normal and reversible consequence of functional plasticity. Secondly, there are calls to replace 'nervous system' with the more stringent 'somatosensory system'. This is the result of doubts over whether neurological disorders that cause pain in the absence of injury (for example fibromyalgia and complex regional pain syndrome) are specifically neuropathic. Thus an updated definition of neuropathic pain, as proposed at the Second International Congress on Neuropathic Pain (Berlin, 2007) by the president of IASP is 'pain due to *disease* of the *somatosensory* system'.

1.2.2 THE CLINICAL PICTURE OF NEUROPATHIC PAIN

Neuropathic pain, as defined above, refers to a heterogeneous set of pain conditions that may occur at any interval along the sensory neuraxis, from peripheral nociceptors to cortical brain areas. The most common locations are the peripheral and dorsal root nerves, with traumatic injuries, entrapment, compression, ischaemia, infection (e.g. by HIV or VZV) and malignant diseases all capable of causing peripheral neuropathic pain. Other common locations are the spinal cord and brain, with spinal cord injuries, stroke and multiple sclerosis all capable of causing central pain. Despite huge diversity in the aetiology and topography of neuropathic pain, symptoms are similar amongst patients and are dominated by a dichotomy of co-existing positive and negative sensory symptoms that correlate with the neuroanatomical distribution (i.e. innervation territory) of the injured nerve (Hansson, 2002). These symptoms, which may occur singly or in various combinations, include partial or total loss of sensation, muscle atrophy and weakness (negative symptoms) as well as static mechanical allodynia (SMA), dynamic mechanical allodynia (DMA), paraesthesias (abnormal sensations), dysaesthesias (abnormal unpleasant sensations), thermal hyperalgesia and after-sensation in the affected area (positive symptoms), as well as other nuisance variables. The vast majority of patients with neuropathic pain are additionally reported to experience spontaneous pain, which may either be constant (described as 'cramping, burning or stabbing') or intermittently paroxysmal (described as 'shooting electric pulse-like').

1.2.3 HUMAN DIAGNOSTIC STUDIES AND RESEARCH

The clinical phenotyping of neuropathic pain depends on certain screening tools, including patient pain questionnaires (Bennett et al., 2007) and bedside sensory examination. A complementary psychophysical approach to assessing the constellation of positive and negative symptoms in patients with neuropathic pain was implemented by the German Research Network of Neuropathic Pain in the form of Quantitative Sensory Testing (QST). This standardised procedure uses calibrated equipment (e.g. brush, von Frey fibres, cold and warm metallic 'Lindblom rollers') to profile the magnitude of sensory deficits, abnormalities and pain detection thresholds in patients with neuropathic pain. Whilst QST is used primarily within the diagnostic

hierarchy of neuropathic pain to define somatosensory functionality and to predict the natural course of pain, it can also be used in a research setting to demonstrate analgesic efficacy with respect to the different components of neuropathic pain (Hansson et al., 2007).

Microneurography is a technique that makes single cell recordings from sensory fibres and is used as a research tool in patients with neuropathic pain to reveal unique patterns of action potential discharge in injured and neighbouring neurones. Pathological findings have revealed spontaneous complex activities in small-diameter touch Aβ fibres, and so-called 'double spike' and spontaneous activities in normally quiescent nociceptive C-fibres. The clinical correlates for these observed abnormalities are thought to be dysaesthesias, hyperalgesia, and spontaneous pain (Bostock et al., 2005, Ochoa et al., 2005). Diagnoses of neuropathic pain in patients may also include nerve biopsies (for visualisation of axonal degeneration) or skin biopsies (to quantify neuronal terminations in the epidermis).

Recent advances in neuroimaging studies in human subjects have gone a long way towards identifying a dynamic pain matrix within the brain (Tracey, 2005). This technique, which uses functional magnetic resonance imaging (*f*MRI) to detect the coupling between oxygen consumption, cerebral blood flow and activated brain areas, has been used to assess whole-brain nociceptive signal processing during the expectation and receipt of painful stimuli (Fairhurst et al., 2007), and has also demonstrated how activity in certain areas can be modulated by different analgesics (Iannetti et al., 2005).

1.2.4 TRANSLATIONAL RESEARCH AND THE NEED FOR SURROGATE ANIMAL MODELS OF NEUROPATHIC PAIN

Human studies in volunteers and clinical studies in patients have greatly contributed towards our understanding of neuropathic pain and play an essential role in shaping the future of research and treatment approaches. However, the tools and techniques used, some of which are mentioned above, are not infallible and are all subject to limitations. There is therefore a need for basic science and surrogate animal models to complement, and in some cases guide, clinical and human research. The

key advantage of animal models that proximate human neuropathic pain conditions is that they enable access to the full spectrum of pathophysiologies in both the peripheral nervous system (PNS) and the central nervous system (CNS) and allow the molecular and cellular events underlying abnormal sensory processing and disproportionate pain to be explored (Le Bars et al., 2001). These pre-clinical models are designed to give a unique understanding of the human condition and can be used to study neurophysiological, morphological and biochemical changes that occur before the onset of clinical signs. Additionally they can be used to assess the pharmacology of the pain state, which can explain the actions of existing analgesic drugs, provide information about efficacy, and identify rational therapeutic targets for future drugs.

Animal models of neuropathic pain must adhere to guidelines set by IASP for the care and use of laboratory animals to minimise suffering (Zimmermann, 1983, Ren and Dubner, 1999). The models can be broadly divided into peripheral mononeuropathy, peripheral polyneuropathy and central neuropathic pain in line with the human situation (see Table 1.1). In my studies I have used the selective spinal nerve ligation (SNL) model of peripheral neuropathic pain in which two of the three left spinal nerves, L5 and L6 are ligated with non-absorbable sutures (Kim and Chung, 1992). This produces partial denervation of one of the hindpaws (so that some sensory input is maintained). Tight ligation of the nerves limits the subjectivity with respect to ligation strength in the other models and therefore limits the extent of inter-rat variation in terms of hindpaw deafferentation. Accordingly, this model produces reproducible, stable and enduring behavioural hypersensitivities in the ipsilateral (injured) hindpaw within two days of surgery, with minimal motor impairment (Yoon et al., 1996, Chapman et al., 1998b). The clinical correlates of this model are thought to be nerve root injury, plexus avulsion injury and possibly nerve compression injury. As with human approaches, animal studies are limited since they predominantly reveal the sensory-discriminative aspect of the complex human pain experience (experimental pain does not reproduce emotional state, setting or past experience for example) and do not provide descriptive information about the quality of the 'pain' and potential side effects of drugs. More recently however, attempts to include measures of the affective-motivational components of pain (for example by using open-field and escape-avoidance paradigms) have been reported (LaGraize et al.,

2004, Hasnie et al., 2007). Regardless of this, animal research abrogates many of the practical and ethical difficulties associated with the measurement of pain in humans, and the models often show good predictive validity, are reliable and reproducible, and have thus far served well in identifying previously unknown causal mechanisms in neuropathic pain that have been extrapolated and translated to the clinic.

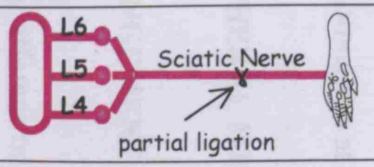
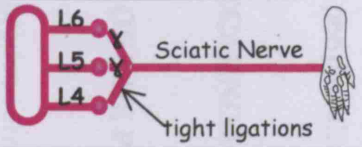
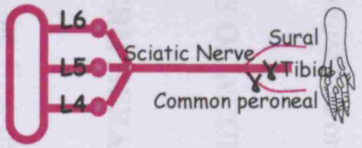
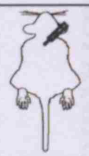
PERIPHERAL MONONEUROPATHY Produces partial denervation of one hindpaw. Some sensory input to the spinal cord is maintained. Typical behaviour characteristics in awake animals include mechanical and cold hypersensitivity in the ipsilateral hindpaw, and attendant nocifensive behaviours, e.g. licking, biting, contact-avoidance foot lifts, guarding and occasional vocalisation	Chronic Constriction Injury (CCI)	Four loose ligatures around the sciatic nerve at the level of the knee joint, proximal to where it branches into L4, L5 & L6 spinal nerves		Bennett & Xie, 1998
	Partial Sciatic Nerve Ligation (PSNL or 'Seltzer' Model)	Tight ligation of a portion of the sciatic nerve so that ~1/2 - 1/3 thickness is ligated.		Seltzer *et al.* 1990
	Selective Spinal Nerve Ligation (SNL or 'Chung' model)	Tight ligation of spinal nerves L5 & L6 distal to the DRG and proximal to the formation of the sciatic nerve		Kim & Chung, 1992
	Spared Nerve Injury (SNI)	Lesion of two of the three terminal branches of the sciatic nerve (tibial & common peroneal, leaving the sural nerve intact)		Decosterd & Woolf, 2000
PERIPHERAL POLYNEUROPATHY Results in behavioural hypersensitivities	Diabetic Neuropathy	Subcutaneous injection of STREPTOZOCIN to induce hyperglycaemia and glucosuria		Ahlgren & Levine, 1993
CENTRAL NEUROPATHIC PAIN Models are supposed to replicate human spinal cord injury caused for example by trauma, ischaemia and radiation	Spinal Ischaemia	Transiently: by occlusion of the descending aorta	Prominent mechanical hypersensitivity localised to the spinal dermatomes that are affected by spinal injury (i.e. hindlimbs and back)	Marsala & Yaksh, 1994
		Permanently: by irradiation		Hao *et al.* 1990
	Excitotoxicity	Inhibit spinal GABA receptors by i.t. injection of strychnine		Yaksh, 1989
		Inhibit spinal $GABA_a$ receptors by i.t. injection of bicuculline		Sherman & Loomis, 1994

Table 1.1 *Animal Models of Neuropathic Pain*

1.3 MECHANISMS OF NEUROPATHIC PAIN

A multiplicity of known and potential mechanisms underlies the complexity of neuropathic pain, with each of the mechanisms, or 'factors', bearing different orders of relevance from one person and pain state to the next. The nervous system's capacity for long-term reorganisation (known as 'plasticity') and neuropathic pain can result from abnormalities at peripheral, spinal and/or supraspinal levels.

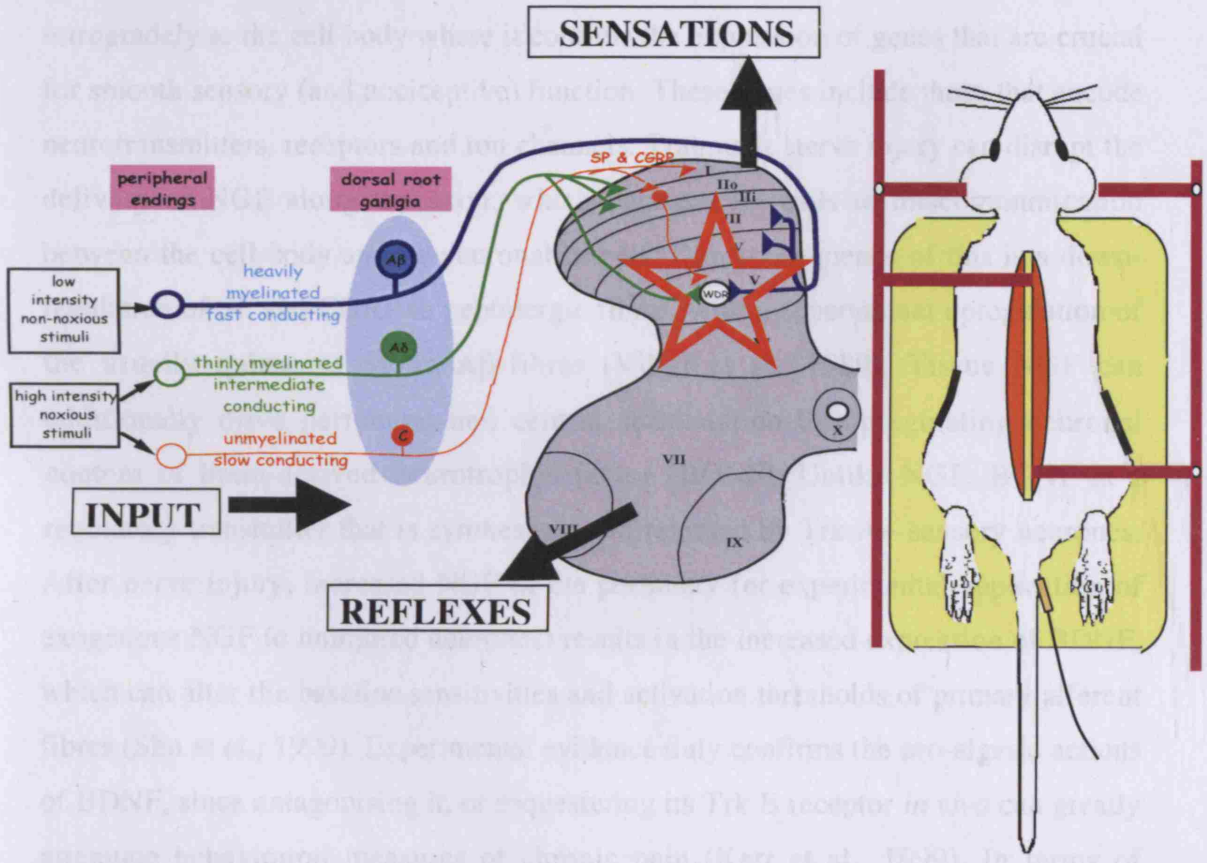
1.3.1 PERIPHERAL MECAHNISMS OF NEUROPATHIC PAIN

1.3.1.1 ARRANGEMENT OF THE PERIPHERAL NERVOUS SYSTEM

Primary afferent neurones form the first relay in the somatosensory chain. They comprise a cell body, which lies in the trigeminal- or dorsal root ganglion (DRG), and stem processes that extend peripherally to their targets (including the skin, deep somatic tissue and the viscera), and centrally to the dorsal horn of the spinal cord (see Figure 1.1 and Section 1.3.2.1). There are essentially 3 types of primary afferent neurones, namely the Aβ-, Aδ- and C-fibres, that can be discriminated anatomically and physiologically, and ultimately by their responsiveness to, and activation by noxious and non-noxious stimuli. Aβ afferents are large diameter, thickly myelinated and therefore fast-conducting fibres (conduction velocity 7-75 m/s) that respond to low-threshold mechanical distortion of their terminals. Aδ afferents are of medium-diameter, are thinly myelinated and conduct action potentials at the intermediate velocity of 2-7 m/s. Typically 1/3 of Aδ-fibres are sensitive mechanoreceptors, conveying innocuous information to the CNS alongside Aβ-fibres, whilst 1/3 are thermoreceptors that respond to noxious heat and cooling, with the remaining 1/3 activated specifically by high intensity mechanical stimuli. The other group of nociceptors, which form the third and final group of primary afferent neurones, are the small-diameter, unmyelinated C-fibres that conduct at the slow velocity of 0.5 – 1.5 m/s. Their polymodal nature means that they respond to a wide range of noxious mechanical, thermal and chemical stimuli. C-fibres can be further segregated into peptidergic and non-peptidergic groups on the basis of their neurotransmitter content: peptidergic C-fibres contain Substance P (SP) and calcitonin-gene-related-peptide (CGRP), express the nerve growth factor (NGF)

receptor Trk A, and as a rule are not involved in pre-synaptic inhibition or synaptic arrangements of a triadic type. On the other hand non-peptidergic C-fibres are involved in these processes, express the $P2X_3$ receptor for ATP, and label positive for IB4 (a plant-derived isolectin). The complex categorisation of C-fibres is extended by the inclusion of 'silent nociceptors' that can be activated by substances released during injury and inflammation. Differences between the populations of nociceptors are likely to be related to different facets of pain detection and transmission, yet the ultimate sensation of pain results from the sum total of signals conducted by different primary afferent fibres that integrate in the dorsal horn of the spinal cord.

Figure 1.1 *Diagram showing the laminar terminations of peripheral Aβ-, Aδ- and C-fibres entering the dorsal horn of the spinal cord. During in vivo electrophysiology, extracellular recordings are made from deep neurones receiving convergent input from Aβ-, Aδ- and C-fibres from the hindpaw.*



1.3.1.2 CONTRIBUTION OF SCHWANN CELLS, GROWTH FACTORS AND PHENOTYPIC SWITCHES TO NEUROPATHIC PAIN

The degree of fibre myelination, which confers fibre conduction velocity, depends on the integrity of enveloping Schwann cells that control sensory neurone development and function. Nerve injury can result in Schwann cell dedifferentiation and a consequent switch from normal myelin production to the dysregulated synthesis of neurotrophic factors. Prolonged exposure of the neuronal environment to excess growth factors can have adverse effects on neighbouring intact and injured neurones, and contribute to the pain phenomenon (Woolf and Salter, 2000, McMahon and Jones, 2004).

The constitutive availability of growth factors in the peripheral targets of large and small diameter sensory neurones maintains normal neuronal phenotype. NGF for example is taken up from its source by free sensory nerve endings and is transported retrogradely to the cell body where it controls the expression of genes that are crucial for smooth sensory (and nociceptive) function. These genes include those that encode neurotransmitters, receptors and ion channels. Traumatic nerve injury can disrupt the delivery of NGF along the axon, which necessarily leads to miscommunication between the cell body and its neuronal targets. One consequence of this is a down-regulation of SP and CGRP in peptidergic fibres, with a concomitant upregulation of the usually quiescent SP in Aβ-fibres (Villar et al., 1989). Tissue NGF can additionally drive peripheral and central sensitisation by upregulating neuronal content of brain-derived neurotrophic factor (BDNF). Unlike NGF, BDNF is a regulatory transmitter that is synthesised and released by Trk A+ sensory neurones. After nerve injury, increased NGF in the periphery (or experimental application of exogenous NGF to uninjured neurones) results in the increased expression of BDNF, which can alter the baseline sensitivities and activation thresholds of primary afferent fibres (Shu et al., 1999). Experimental evidence duly confirms the pro-algesic actions of BDNF, since antagonising it, or sequestering its Trk B receptor *in vivo* can greatly attenuate behavioural measures of chronic pain (Kerr et al., 1999). In terms of treatment, surrogate sources of the neurotrophic factor GDNF have been shown to have potent neuroprotective effects on axotomised sensory neurones, can prevent mechanical sensitivities that develop after SNL surgery, and can reverse some of the

changes in Na^+ channel expression that are consequent to Schwann cell disorganisation and neuropathic pain (Boucher et al., 2000).

1.3.1.3 ION CHANNELS UNDERLYING NEUROPATHIC PAIN AND THEIR THERAPEUTIC POTENTIAL

Successful treatment of neuropathic pain is contingent on a good understanding of underlying mechanisms. Ion channel plasticity is known to intimately link with neuropathic pain, hence many analgesic drugs have been designed (sometimes serendipitously) to modulate these channels. In particular, channels that are voltage-gated for Na^+, K^+, and Ca^{2+} ions (as well as the NMDA receptor-complex) underlie the processing of sensory information and are key targeting candidates. There is, in addition, increasing interest in the therapeutic potential of $P2X_3$ receptor-gated ion channels and the so-called hyperpolarisation-activated cyclic nucleotide-modulated (HCN) "pacemaker" channels, both of which appear to play a pathophysiological part in certain pain states.

Voltage-Gated Na^+ Channels

Voltage-gated Na^+ channels propagate action potentials along neurones and spur hyperexcitability after nerve injury. Different voltage-gated Na^+ channel isoforms, with different kinetic and pharmacological properties, have been delineated in sensory neurones. The $Na_v1.8$ and 1.9 α subunits are expressed exclusively in small, unmyelinated fibres and are resistant to block by tetrodotoxin (TTX), whilst the $Na_v1.7$ α subunit, which is susceptible to block by TTX, is expressed in sensory *and* sympathetic neurones. Tissue and nerve damage can lead to a change in the expression and function of α subunits, and a resultant change in neuronal excitability to the detriment of the sensory system (Okuse et al., 1997, Kim et al., 2001, Strichartz et al., 2002). Tellingly, inherited 'gain of function' mutations in the $Na_v1.7$ α subunit in humans result in erythermalgia, a painful condition characterised by intolerable burning sensations in the extremities (Yang et al., 2004), whilst other mutations in this channel result in paroxysmal extreme pain disorder.

Peripheral nerve damage leads to a downregulation of 1.8 and 1.9 transcripts in the DRG (despite the translocation, insertion and clustering of Na^+ channels

containing these subunits at injury and neuroma sites) (Kim et al., 2001), with a concomitant upregulation of the embryonic TTX-s α 1.3 subunit. This subunit is usually only expressed in developing sensory neurones, hence its re-expression is associated with nerve damage and increased neuronal excitability (Hains et al., 2003). The newly dense distribution of Na^+ channels along the sensory neurone after nerve injury supports ectopic firing whereby action potentials propagate along the neurone in the absence of a stimulus. Such spontaneous discharges, which result partly from Schwann cell de-differentiation as mentioned above, can be replicated experimentally by the addition of a de-myelinating agent (the detergent lycosolecithin) to Aδ fibres (Wallace et al., 2003), and are known to promote cross-talking between damaged and uninjured fibres by means of ephatic communication. Various studies suggest that ectopic activities in sensory neurones are mediated by $Na_v1.3$ α subunits, and not 1.7, 1.8 or 1.9 α subunits (interestingly, neuropathic pain develops normally in mice lacking $Na_v1.7$ and/or 1.8 subunits despite their known involvement in setting mechanical sensory thresholds) (Nassar et al., 2005). Na^+ channels with the 1.3 subunit have the correct biophysical properties to support rapid firing, are upregulated in all models of neuropathic pain, and spontaneous firing can be blocked by TTX.

TTX blocks Na^+ ion transport across the membrane by directly occluding the channel pore in a tonic fashion. Other channel modulators compromise ion flow under specific (patho)physiological conditions by virtue of state-dependence. This is the principle that underlies the analgesic capacity of anti-epileptic drugs such as carbamazepine and the structurally related oxcarbamazepine. These agents slow the recovery of rapidly firing voltage-gated Na^+ channels in a frequency-dependent way, and have been shown to be effective in the treatment of trigeminal neuralgia (with observations of efficacy dating back to 1965 (Burke et al., 1965)), diabetic neuropathy and post-herpetic neuralgia (Criscuolo et al., 2004, Beydoun et al., 2007). Lamotrigine, another anti-epileptic agent, works in a similar way, whilst the anti-arrhythmic mexilitine, as well as the local anaesthetic lignocaine, non-selectively block voltage-gated Na^+ channels to ease neuropathic symptoms (the latter usually applied as a 5% topical patch), regardless of the channel's location in the body. The side-effects of these agents relate to their lack of selectivity within the sodium channel family, so the recent development of a selective 1.8 blocker, shown to be effective in preclinical models of neuropathy, may be key in improving the therapeutic index of

drugs acting on sodium channels (Jarvis et al., 2007).

Voltage-Gated K^+ Channels

In contrast to voltage-gated Na^+ channels, voltage-gated K^+ channels act as brakes in the system, repolarising active neurones to restore baseline membrane potentials. K_v7 (KCNQ) channels operate at low thresholds in the CNS, and are responsible for the inhibitory M current in DRG neurones. Retigabine is an agent that facilitates the M current through the opening of K_v7 channels, and has accordingly been shown to inhibit electrically-evoked dorsal horn neuronal responses *in vivo* in a dose-dependent manner. Moreover, retigabine's actions are retained, if not enhanced after nerve injury (despite alterations in K^+ channel expression) hence there is interest in the development of this opener as an analgesic that can be used in the mainline treatment of neuropathic pain, in addition to openers that operate at other potassium channels, for example those belonging to the SK family (Bahia et al., 2005).

HCN Channels

Bearing significant structural homology to K^+ channels are the HCN channels, which are permeable to both K^+ *and* Na^+ ions. These channels prevail in cardiac tissue and DRG neurones where they modulate the rhythm and waveform of action potentials and contribute to resting membrane potentials (Mayer and Westbrook, 1983, Robinson and Siegelbaum, 2003). Nerve trauma induces a parallel increase in HCN open channel probability and the proportion of primary sensory neurones showing a pacemaker current, which likely contributes towards enhanced transmitter release in the spinal cord after nerve injury. Accumulating evidence points towards an important role of HCN channels in A-fibre mediated mechanical 'allodynia' and spontaneous neuronal discharges associated with peripheral nerve injury; Systemic (but not intrathecal) injection of a specific blocker has been shown to dose-dependently suppress behavioural hypersensitivities in SNL rats, whilst extracellular recordings *ex vivo* from dorsal root fibres have demonstrated the efficacy of this blocker in reducing spontaneous A-fibre discharge. Importantly, these changes were observed in the absence of adverse effects (i.e. cardiotoxicity and death), and left normal sensory transduction intact (Chaplan et al., 2003, Luo et al., 2007).

Voltage-Gated Ca^{2+} Channels

Voltage-gated Ca^{2+} channels conduct Ca^{2+} ions into the neurone during depolarisation, and therefore play a role in synaptic transmitter release, membrane excitability and intracellular signalling events that can alter gene expression and lay the foundations for long-term-potentiation (LTP). The sensory neurone's repertoire of voltage-gated Ca^{2+} channels includes L-, N-, P/Q-, R- and T-type channels which subserve different functional roles relative to their cellular locations. L channels, like voltage-gated Na^{+} channels, are key determinants of membrane excitability, whilst N and P/Q channels are involved with transmitter release at synaptic junctions. Supporting this release are the R channels, which are particularly prevalent in nociceptive spinal cord pathways. T channels are low voltage-activated channels that permit Ca^{2+} flux at resting membrane potentials, hence their role in pacemaking, neuronal bursting and synaptic signal boosting. Various experiments point to an altered role of voltage-gated Ca^{2+} channels after neuropathy, and specific blockers have been shown to differentially attenuate the behavioural hypersensitivities and altered dorsal horn neuronal responses that accompany experimental neuropathic pain (Matthews and Dickenson, 2001a, Matthews and Dickenson, 2001b, Matthews et al., 2007).

Regarding the serendipity of drug discovery, a prime example is gabapentin (GBP), which, in its design as a lipophilic analogue of the inhibitory transmitter GABA was intended as an anti-epileptic agent but was actually found to bind to the $\alpha_2\delta$ subunit of the voltage-gated Ca^{2+} channel to reduce Ca^{2+} entry into the neurone, thereby attenuating transmitter release. This qualifying property means that GBP, as well as its structural isomer pregabalin (PGB) (thought to be a more pharmaceutically elegant agent) are considered good starting options in the treatment of many neuropathic pain conditions.

In cases of limited GBP/PGB efficacy, these agents can be paired with morphine, which itself reduces both Ca^{2+} entry into the neurone and subsequent transmitter release via actions at pre-synaptic mu-opioid receptors. Opioids have remained the gold standard of pain control for many thousands of years. They have multiple sites of action (see later) and can either be given synergistically as described (Matthews and Dickenson, 2002), or independently so long as they maintain

acceptable side-effect profiles.

Centrally Located NMDA Receptor-Complexes

Ca^{2+} ions additionally gain entry into neurones via NMDA receptor-complexes, which require dual activation by the excitatory transmitter glutamate and depolarising membrane potentials. This reliance on coincident pre- and post-synaptic activity (which affords them the term 'coincidence detectors') ensures that the receptors are only activated, and the Ca^{2+} channel opened, during intense synaptic activity. These complexes are involved in central sensitisation (Dickenson and Sullivan, 1987, Dickenson, 1990) and their expression and docking at synaptic sites increases after nerve injury. However, the multiple roles of NMDA receptors in normal and abnormal nervous system function (memory and learning v. pathological pain processing) impose important constraints on therapeutics that block or modulate their conductance. For example, in a randomised controlled trial, 57% of patients with chronic pain that were being treated with sub-anaesthetic doses of ketamine discontinued treatment within a week due to side-effects including sedation and dizziness (Haines and Gaines, 1999). Pharmacological properties of channel modulators can however be refined so that agents preferentially bind to open channels, conferring a pathologically activated state that has superior tolerability. This is the mechanism of action of memantine and nitromemantine, agents that are currently being trialled for the treatment of neuropathic pain. Given that nerve injury preferentially upregulates the expression of the NR2B subunit and that this expression can be blocked by pre-emptive NMDA antagonism (Wilson et al., 2005), an additional therapeutic avenue being explored is the selective (and pre-emptive) modulation of these particular subunits by agents such as ifenprodil (Iwata et al., 2007).

$P2X_3$ Receptor-Gated Ion Channels

$P2X_3$ receptors are abundantly expressed in large unmyelinated sensory neurones where they gate cation channels and mediate ATP nociceptive signalling. The relationship between these receptors, whose expression increases after nerve injury, and neuropathic pain has been confirmed using antisense against $P2X_3$ mRNA; when the oligonucleotides are injected into lumbar segments of the spinal cord, they significantly attenuate both the development of nerve injury-induced behavioural

hypersensitivities and established pain behaviours (Barclay, 2002).

Future trends in pain-related ion channel research are likely to involve deconstructions of native ion channel compositions, distributions, interactions and polymorphisms, which will hopefully lead towards increased target validation (and elimination), subtype selective pharmacology, and ultimately effective analgesia.

1.3.1.4 SYMPATHETIC SUPPORT OF PATHOPHYSIOLOGICAL PAIN

ATP, which activates the above-mentioned $P2X_3$ receptors in the DRG during neuropathic pain, could conceivably come from sympathetic neurones that invade sensory fibres after nerve injury, weaving basket structures around their cell bodies so that they are forced together in close proximity (Chung et al., 1997). This coupling of the otherwise anatomically discrete sympathetic and sensory nervous systems can enhance spontaneous and evoked neuronal activity and recruit silent nociceptors, providing a functional correlate for sympathetically-maintained pain (SMP). SMP has been identified in some clinical cases of neuropathic pain, with some neuropathic patients showing atypical symptoms such as excessive sweating and vasomotor anomalies. In these patients, painful symptoms can be intensified by stimulating the sympathetic system, relieved by the noradrenaline-depleting agent guanethidine, and rekindled by intra-dermal administration of adrenergic agents (Torebjork et al., 1995).

1.3.1.5 PRIMARY AFFERENT FIBRE REORGANISATION

In addition to sympathetic interference, two other forms of erroneous sprouting have been proposed as mechanisms in neuropathic pain. The first of these hypotheses concerns the peripheral nervous system and its regenerative capacity; following nerve transection, Schwann cells accrue and promote neuronal re-development, which can result in de-afferentation of injured and uninjured fibres. This 'collateral sprouting' as it is known, can lead to mis-directed targeting of fibres and inappropriate peripheral innervation such that cutaneous areas once occupied by the lesioned nerve becomes hyper-innervated by low- and high-threshold fibres. The behavioural manifestation of this sprouting, which has been demonstrated in skin samples from neuropathic patients, includes touch-evoked pain.

Whether or not peripheral reorganisation of the nervous system after nerve injury is matched by central reorganisation in the spinal cord has been the subject of considerable debate. This follows seminal work suggesting that central terminals of myelinated afferents redistribute after nerve injury into dorsal horn laminae, which, in the normal state are exclusively occupied by the central terminals of unmyelinated neurones (Woolf et al., 1992). This purported shift in fibre termination would enable low-threshold sensory fibres to access nociceptive-specific relay neurones, meaning that innocuous stimuli would be inappropriately translated and painfully perceived. Whilst this provides a tidy explanation for the hyperaesthetic pathophysiologies associated with neuropathic pain, results have been confounded by subsequent observations that the bulk-labelling procedure indiscriminately labels the terminals of both myelinated *and* unmyelinated fibres (and not just myelinated fibres as originally thought). Support for this central sprouting hypothesis has therefore abated, and it is now thought that any potential dorsal horn reorganisation following nerve injury is markedly less significant than once thought.

1.3.1.6 PERIPHERAL SENSITISATION

If allodynia is not a consequence of low-threshold Aβ-fibres tapping into an altered CNS after nerve injury, it may alternatively be caused by a reduction in the thresholds of nociceptor terminals in the periphery. Peripheral sensitisation is mediated by a range of inflammatory substances, including prostaglandins, cytokines and histamine that are released during previous or actual injury. These mediators can evoke the axon reflex (resulting in vasodilation and neurogenic oedema formation) and increase the excitability of nociceptors so that they activate at lower thresholds. Sensitisation can additionally result in an expansion of the sensory neurone's peripheral receptive field, and cause an acceleration in firing rate. The barrage of activity that follows can increase neuronal transmitter release and unleash NMDA receptors in the dorsal horn of the spinal cord, triggering central sensitisation (Dickenson, 1995a).

1.3.2 CENTRAL MECHANISMS OF NEUROPATHIC PAIN

Central sensitistion is an umbrella term that refers to the various mechanisms underlying the disproportionate, augmented responses of dorsal horn neurones to incoming sensory input.

1.3.2.1 DORSAL HORN REPRESENTATION OF SENSORY INPUT

The central terminals of primary afferent fibres show highly organised, topographic termination patterns in the dorsal horn of the spinal cord. The dorsal horn houses the cell bodies of 2nd order sensory neurones, and together with the ventral horn, forms the grey matter of the spinal cord. Cross-sections of the dorsal and ventral horns that run the entire length of the spinal cord have been divided into horizontal layers, or 'laminae', according to Rexed's classification based on morphological features (Rexed, 1952). These laminae number I through to IX in a dorso-ventral direction, and there is an additional column of lamina X cells that cluster around the central canal. Afferent neurones arriving from the periphery enter the spinal cord via dorsal root entry zones and terminate in specific laminae in keeping with their neuronal type and sensory signal (see Figure 1.1 above).

Lamina I is a thin 'marginal' layer that receives synaptic input from small-diameter unmyelinated C-fibres and finely myelinated Aδ-fibres. It comprises small neurones that are largely nociceptive (some being nociceptive specific – NS), with a smaller number of neurones being thermoreceptive or sensitive to itch stimuli. Many lamina I neurones project long-distance axons to the brain along designated tracts (for example the spino-parabrachial tract), whilst other neurones send dendrites to local neurones within the same lamina, or dorsally to distal neurones in other laminae. Lamina I neurones can modulate the excitability of neurones in deeper laminae directly via intraspinal pathways, or indirectly in loop form via pathways that descend from brainstem areas (more later) (Suzuki et al., 2002b, Suzuki et al., 2003).

Lamina II, known as the 'substantia gelatinosa' is where nociceptive interneurones involved in the processing of noxious input are principally located. Accordingly, fibres conveying noxious input from the periphery terminate here; the

outer aspect of lamina II (II_o) receives input from Aδ-fibres and IB4+ non-peptidergic C-fibres, whilst the more basal, inner aspect (II_i) receives the terminals of TrkA+ peptidergic C-fibres. Like lamina I cells, neurones within the substantia gelatinosa can send projections beyond their segmental area to modulate neuronal activity in other laminae (i.e. I and IV).

Laminae III and IV are collectively known as the 'nucleus propius'. They contain cells that respond to innocuous input such as that arriving along Aβ-fibres.

Lamina V, which is set deep in the dorsal horn, receives convergent input from low- and high-threshold sensory fibres. The ability of neurones in lamina V to amalgamate sensory input ranging from the innocuous to the noxious affords the prefix 'wide dynamic range' (WDR). WDR neurones have a high propensity for wind-up (so that upon repeated stimulation they temporally summate incoming action potentials and react to given stimuli with exaggerated response) and have large receptive fields. This is in contrast to NS neurones in superficial laminae that have small receptive fields and undergo minimal wind-up in response to repetitive stimulation. Following nerve injury, NS neurones may take on characteristics of WDR neurones and therefore become responsive to a more spatially and sensory diverse range of stimuli (Suzuki et al., 2000). WDR neurones can project beyond lamina V boundaries (as well as projecting locally within their own segment) and can additionally send axons along ascending tracts to supraspinal areas. For example, a proportion of WDR neurones project up the spino-thalamic tract to the reticular formation and thalamus. Here, neuronal signals are further processed, before being relayed to cortical areas of the brain whereupon they are decoded into sensory-discriminative and affective-motivational components.

Lamina VI receives input from the afferents of specialised muscle structures, which may also penetrate and make synaptic contact in the ventral horn of the spinal cord. Laminae VII – IX, which form the ventral horn, are where the cell bodies of motor neurones concentrate. Simple synaptic contact between incoming sensory neurones and motor neurones located here can effect reflex responses via striatal muscles, including the nocifensive withdrawal reflex that serves to unconsciously protect an organism from impending injury.

1.3.2.2 DORSAL HORN MODULATION OF SENSORY INPUT: ADVANTAGES & DISADVANTAGES

The integrative milieu of the dorsal horn promotes vast modulation of the sensory signal on its journey from the periphery to the brain. The signal is gated here (Melzack and Wall, 1965) and can either be dampened by the inhibitory system, or enhanced by the excitatory system (Dickenson, 1996, Dickenson, 2002). Short-term signal amplification can cause wind-up as described, whilst prolonged amplification can result in LTP and chronic pain (Bliss and Lomo, 1973).

With respect to sensory signals becoming inhibited, analgesia can be recruited as part of an organism's defence or coping strategy. Stress-induced analgesia for example is an adaptive response whereby inhibitory systems are activated and anti-nociception is endogenously evoked by innate or learned warning cues. This means that pain behaviours are subordinated to more urgent needs of the individual, which may include escaping the conflict and ultimately survival. On the other hand, sensory input may be tuned in the spinal cord so that the signal: noise ratio increases. This forces the individual to attend to their pain and seek relief. Maladaptive amplification may however ensue, triggering processes that ultimately sustain the pain so that it outlasts the time taken for precursory injuries to heal. Thus in this circumstance, central sensitisation switches so that it no longer depends on nociceptor activity, becoming dependent instead on spinal cord activity and supraspinal events. This may be important in central pain states caused for example by stroke and spinal cord injuries. Indeed, increased expression of $Na_v1.3$ Na^+ channels in second-order spinal dorsal horn neurones, and third-order thalamic neurones in these pain states are thought to amplify hyperexcitability in nociceptive pathways, thus contributing to their maintenance (Waxman and Hains, 2006).

1.3.2.3 DISINHIBITION AS A MEANS OF SIGNAL FACILITATION

After peripheral nerve injury there is a significant loss of afferent fibres reaching the dorsal horn of the spinal cord (Castro-Lopes et al., 1990), yet remarkably, dorsal horn traffic is maintained and synaptic signals are invariably enhanced (Suzuki et al., 2001). This is due to compensatory changes in remaining

sensory neurones, particularly C-fibres (Wall et al., 1982), and also due to changes in a newly wired and mal-shaped CNS.

The abolition of steady pre-synaptic inhibitions after nerve injury, for example due to down-regulation of pre-synaptic μ-opioid receptors (Kohno et al., 2005), can be matched by significant reductions in central post-synaptic inhibitions which are partly the result of reduced expression of inhibitory GABA receptors on dorsal horn neurones (Dickenson et al., 1997, Torsney and MacDermott, 2006). There is, in addition, selective apoptosis of inhibitory GABAergic interneurones in the dorsal horn after nerve injury, thought to be due to activity-dependent excitotoxicity (Sugimoto et al., 1990, Moore et al., 2002). The significant upregulation of neuropepetides and EAAs in primary afferent neurones after nerve injury, coupled with increased release and expression of post-synaptic NK1, AMPA and NMDA receptors (Harris et al., 1996, Kawamata and Omote, 1996) means that there is constant depolarisation of some dorsal horn cells. The resultant surge in intracellular Ca^{2+} phosphorylates and primes a host of enzymes to initiate signalling cascades that eventually end with fragmented DNA and cell death. Caspase enzymes are involved in this suicide programme, and if their activity is blocked after nerve injury with the caspase-specific inhibitor ZVAD, the dramatic injury-induced loss of neurones in the superficial dorsal horn is prevented (Scholz et al., 2005). Moreover, this compound, which is not analgesic *per se*, can reduce mechanical hypersensitivities and pain behaviours associated with experimental nerve lesions. There are also shifts in brainstem-derived modulatory controls (which will be described in more detail below in Section 1.3.3) which can further destabilise the balance between excitation and inhibition in favour of the former, meaning that inordinate 'pain' signals are un-gated in the spinal cord and transmitted up to the brain.

1.3.2.4 IMMUNE SYSTEM INVOLVEMENT IN NEUROPATHIC PAIN

Immune system products such as TNFα may additionally trigger caspase-signalling pathways resulting in cell death. The immune system plays an obvious role in inflammatory pain (McMahon et al., 2005), yet its previously underestimated part in neuropathic pain is being increasingly recognised (Marchand et al., 2005). The loss of trophic support after nerve injury has profound effects on gene expression in

neuronal and non-neuronal cells. In the periphery, newly expressed cytokines are released from injured nerves, and chemotractant signals are released from Schwann cells, resulting in sequential macrophage infiltration in the damaged area. These themselves release pro-inflammatory agents, resulting in further macrophage recruitment and product release, sustaining the cycle of nociceptor sensitisation and altered trophic support. Microglia are the resident immune cells in the CNS (functionally equivalent to peripheral macrophages), and together with astrocytes, constitute the glial population. Glia entangle with central neurones and play a key role in pathophysiological pain (Garrison et al., 1991).

Fractalkaline is a neurone-to-glia signal that is ordinarily tethered to sensory neurones (Chapman et al., 2000), yet excessive neuronal activity, such as that seen during neuropathy, can break the attaching stalk releasing fractalkaline into the extracellular fluid. From here it binds to microglia and activates the release of neuro-stimulating agents such as NO, ATP, EAAs and classical immune mediators, inducing spinal nociceptive facilitation (Milligan et al., 2005). The predictive relationship between glial activity and hyperalgesia can be demonstrated with flucocitrate, an agent that blocks glial activation and thus pain-related behavioural hypersensitivities. Similarly, intrathecal delivery of minocycline, or systemic propentofylline, compounds that inhibit microglia activation, reduces the development of nerve-injury induced hypersensitivities, yet does not reverse established pain behaviours (Raghavendra et al., 2003, Tawfik et al., 2007). This is also true of etanercept, a TNFα-sequestering agent that is used to treat the pain associated with rheumatoid arthritis. On a similar note, pre-clinical studies have shown that intrathecal ibuprofen dose-dependently attenuates hyperalgesia via inhibitory actions on spinal cord COX2 isoforms (N.B. actions are not the result of re-delivery to the periphery) (Lucas et al., 2005), yet neuropathic patients remain equivocal responders to COX inhibitors. Nevertheless, given that glial cells are not involved in normal pain processing and only activate during excessive nervous system activity, it is hoped that agents targeting these cells, or their neuroactive products, will hold increased analgesic hope for the future.

1.3.2.5 INITIATION VERSUS MAINTENANCE OF NEUROPATHIC PAIN

An interesting concept that is emerging from several independent studies, including some already mentioned, is that there are separate, albeit widely overlapping processes that underlie the initiation and maintenance of neuropathic pain and their associated behaviours. The examples cited above concern the central immune system and its preferential involvement in the initiation of neuropathic pain (Xu et al., 2006). This hypothesis has been explored in a different context, at the level of the brainstem with resultant conclusions suggesting that this areas is involved in the maintenance, but not initiation, of neuropathic pain (Porreca et al., 2001, Burgess, 2002).

1.3.3 SUPRASPINAL CONTROL OF SPINAL SENSORY PROCESSING

Complex networks of pathways from various sites in the brain integrate together to modulate the spinal processing of sensory information in a top-down fashion. Higher order cognitive and emotional processes such as anxiety, mood and attention can influence the perception of pain through the convergence of somatic and limbic systems into a so-called descending modulatory system, providing a neural substrate through which the brain can control pain.

1.3.3.1 THE PLACEBO EFFECT ("I SHALL PLEASE")

The brain's ability to issue some control over pain is the principle that underlies the placebo effect. This refers to the variable capacity of an inert substance to produce some beneficial (i.e. pain killing) effect with no pharmacological (or related) basis to do so. Crucially, the success of placebo depends on the subject's conscious perception of the therapeutic intervention, with improvement attributable to the expectation of relief. *f*MRI scans have identified common neural mechanisms shared by placebo analgesia and opioid analgesia, and interestingly, activity in these areas can be suppressed in both circumstances by naloxone (Benedetti et al., 1999, Petrovic et al., 2002). Numerous tests and imaging studies have shown that the analgesic placebo effect is at least partly mediated by descending systems that use endogenous opioids as neuromodulators. Co-variant activity between different

brainstem areas (which are known to be rich in opioid receptors) during placebo emphasise the role of the brain in pain modulation.

1.3.3.2 ANATOMICAL CIRCUITRY THAT LOOPS TOGETHER THE SPINAL CORD, MIDBRAIN AND BRAINSTEM AREAS (Fields et al., 1991) (Gauriau and Bernard, 2002) (Suzuki et al., 2002b) (Mason, 2005)

A schematic and highly simplified diagram showing the anatomical connections between the spinal cord, midbrain and brainstem areas is given in Figure 1.2. As can be seen, there are parallel pathways that project from spinal cord laminae to the brain. These pathways are anatomically and functionally separate, yet collectively they and their targets manage to elaborate the nociceptive signal into a complex pain experience.

Lamina I Projections

Two main pathways connect lamina I nociceptive projection neurones with the brain. The first of these is the spino-parabrachial pathway (Bester et al., 1997), which as the name suggests, terminates in the parabrachial area where numerous inputs converge onto significantly fewer cells (Flink et al., 1983). The parabrachial area collects and integrates far-ranging nociceptive signals, before processing them and distributing them onwards (Hayes et al., 1984, Blomqvist et al., 1989). Its key output areas are the amygdala, hypothalamus and periaqueductal grey (PAG). The amygdala triggers emotional affect, including aversion, anxiety and fear-induced avoidance learning (Davis, 1986, Maren, 2007), whilst the hypothalamus aligns homeostatic processes (e.g. blood pressure and heart rate) to the painful input (Vidal et al., 1984). In turn the PAG triggers emotional behaviours that include passive and active coping strategies (withdrawal from the environment versus engagement) (Lovick, 1985), as well as opioid and non-opioid endogenous analgesia (Lewis and Gebhart, 1977). Thus this pathway serves the affective-motivational dimension of pain (Saper, 1995). The other main pathway from lamina I (which is less dense than the spino-parabrachial pathway) projects to caudal parts of the thalamus, which itself projects to the insular and somatosensory cortices for sensory discrimination and interoception (Bester et al., 2000). Lamina I cells that terminate in the thalamus are particularly adept at localising the nociceptive stimulus, and this is largely due to their restricted peripheral receptive

fields (Bester et al., 2000).

In summary, dual pathways that link lamina I nociceptive projection neurones with the brain (particularly the parabrachial area and the thalamus) subserve affective-motivational and sensory-discriminative roles in the pain experience.

Lamina V Projections

Like lamina I projection neurones, neurones from the deep dorsal horn project to one of two major sites, namely the thalamus (hence the spino-thalamic pathway) and the caudal reticular formation. The thalamus projects onto the prefrontal cortex, which is involved with attention and motivational aspects of pain (and therefore would play an important role in people that overly attend to, and catostrophise, their pain) (Wiech et al., 2005). The caudal reticular formation on the other hand underlies the motor response to pain, whilst its intrinsic subnucleus reticularis dorsalis processes the quality, modality and intensity of the incoming stimulus (Villanueva et al., 1991). Lamina V neurones additionally project to the striatum and globus pallidus.

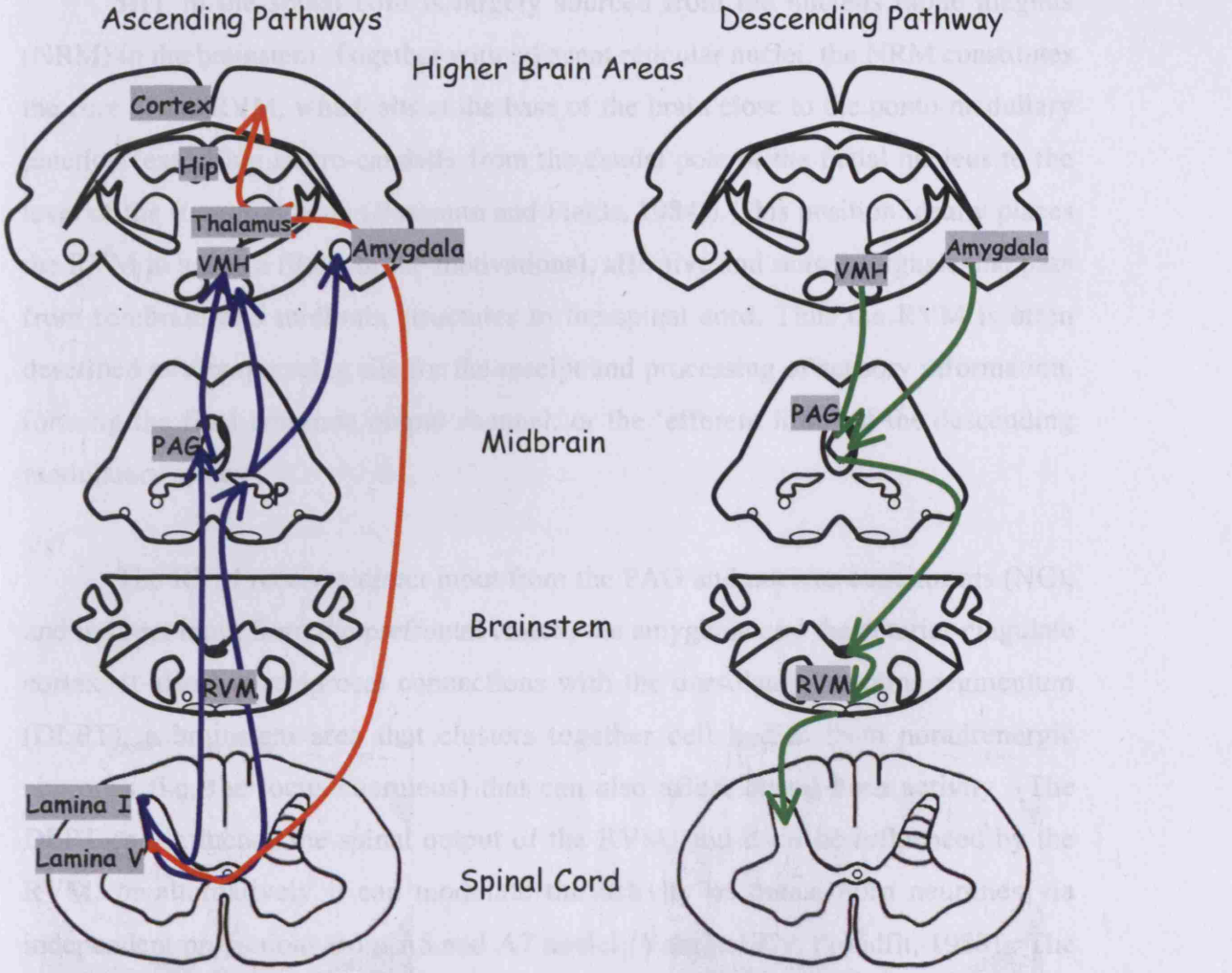
Thus deep laminae projection neurones play a large role in somatic responses and sensory decoding, and via thalamo-cortical projections, a lesser role in the emotional coupling of the painful stimulus.

Lamina I and lamina V pathways can be separated from one another by targeted ablation techniques using the toxin saporin attached to the NK1 receptor agonist Substance P (Mantyh et al., 1997). The conjugate is injected into the spinal cord whereupon it internalises into NK1 receptor-expressing lamina I projection neurones via G protein-coupled receptor internalisation. Intracellularly, the receptor, Substance P and saporin dissociate, allowing saporin to interfere with ribosomal machinery which eventually destabilises the cell and causes its death. Disrupting lamina I projection pathways in this way produces only a marginal shift in acute pain sensitivity, yet in circumstances of ongoing pain, such as that caused by neuropathy, spinal excitability and the plasticity of the response to injury are affected (Khasabov et al., 2002). This is largely because spinal-brainstem modulatory pathways become disabled as a result of the lamina I lesions.

Feedback Regulation of Sensory Information

There is significant feedback regulation of the nociceptive signal courtesy of a spino-bulbo-spinal loop which first projects up from the spinal cord to the brain as described, before descending back down to the spinal cord via brainstem structures to alter subsequent incoming signals (Suzuki et al., 2002b). One consequence of this in the context of neuropathic pain is that nociceptive information can precipitate supraspinal neuroplastic changes to further facilitate incoming spinal inputs, resulting in an enhanced pain state. NK1 receptor-expressing lamina I neurones lie at the origin of this loop and indirectly drive changes in deep dorsal horn neurones via this midbrain-reaching circuit. These changes are mediated, at least in part, by serotonin (5HT) acting on facilitatory $5HT_3$ receptors (Suzuki et al., 2002b).

Figure 1.2 *The Main Ascending and Descending Pathways (adapted from Hunt & Mantyh, 2001)*



Spinoparabrachial pathway:
Originates from the superficial dorsal horn and projects to areas of the brain concerned with affect

Spinothalamic pathway:
Originates from deep dorsal horn and projects to areas of the cortex concerned with discrimination and affect

Hip - Hippocampus

PAG - Periaqueductal Gray

PB -Parabrachial Area

RVM - Rostroventral Medial Medulla

VMH - Ventral Medial Nucleus of the Hypothalamus

1.3.3.3 THE ROSTRAL VENTROMEDIAL MEDULLA (RVM)

5HT in the spinal cord is largely sourced from the nucleus raphé magnus (NRM) in the brainstem. Together with adjacent reticular nuclei, the NRM constitutes the core of the RVM, which sits at the base of the brain close to the ponto-medullary junction (extending rostro-caudally from the caudal pole of the facial nucleus to the level of the trapezoid body (Basbaum and Fields, 1984)). This position ideally places the RVM to act as a filter for the motivational, affective and sensory signals that pass from forebrain and midbrain structures to the spinal cord. Thus the RVM is often described as a major relay site for the receipt and processing of sensory information, forming the final common output channel, or the 'efferent limb' of the descending modulatory system.

The RVM receives direct input from the PAG and nucleus cuneiformis (NC), and indirect input from the prefrontal cortex, the amygdala and the anterior cingulate cortex. It also has reciprocal connections with the dorsolateral pontine tegmentum (DLPT), a brainstem area that clusters together cell bodies from noradrenergic neurones (i.e. the locus coeruleus) that can also affect dorsal horn activity. (The DLPT can influence the spinal output of the RVM, and itself be influenced by the RVM, or alternatively it can modulate the activity of dorsal horn neurones via independent projections from A5 and A7 nuclei (Yaksh, 1979, Proudfit, 1988)). The RVM sends neurones down the dorsolateral and ventrolateral funiculi (DLF) (VLF) to the spinal cord where synapses are made with terminals of primary afferent neurones, ascending tract neurones, intrinsic interneurones and the terminals of other descending neurones. Anterograde labels and antidromic stimulation have identified intense RVM innervation of laminae I, II and V (Basbaum, 1981, Skagerberg et al., 1985, Fields et al., 1995a) which are all territories occupied by the central terminals of peripheral nociceptive neurones. This at once suggests the (proven) role of the RVM in nociceptive modulation. The RVM does not however exclusively serve nociception since its neurones also project to circumcanular lamina X neurones where they modulate parasympathetic and sympathetic outflow and have eventual effects on autonomic targets (e.g. the heart, cutaneous blood vessels and the adrenal medulla) in conditions unrelated to pain. Ultimately though, the RVM's modulation of nociception may trump other functions and supersede autonomic responses, causing

them to react in line with the painful stimulus (for example by increasing heart rate and blood pressure).

RVM Output Neurones

In conjunction with the PAG in the brainstem, the RVM gives rise to pathways that differentially engage descending facilitatory and inhibitory neurones to respectively increase and decrease dorsal horn activity. The recruitment of these neurones can alter under different conditions and circumstances such that sensory transmission and ultimately pain perception may be potentiated or suppressed. The neural basis for this bi-directional modulation has long been established; in the 1980s, Fields and colleagues heuristically categorised 3 groups of RVM cells according to their physiological responses to cutaneous nociceptive stimulation (Fields et al., 1983a). 'On' cells reliably showed a burst of activity immediately prior (~400ms) to a reflex paw/tail withdrawal to noxious heat, whilst 'Off' cells exhibited a pause in firing before the same stimulus-evoked response (see Figure 1.3). A third group of cells were labelled 'neutral' on the basis that they showed little or no consistent change in firing related to the nocifensive withdrawal response. The role of neutral cells in nociception remains unclear and is the subject of ongoing investigation (more later).

It was also shown in the same and related studies, that the responses of On and Off cells to peripheral noxious stimuli are inversely predictive of their responses to systemic or local opioid administration (Fields et al., 1983b, Barbaro et al., 1986). Thus On cells are inhibited by morphine and Off cells are activated. This led to proposals that On cells exert a net facilitatory effect on spinal transmission, whilst Off cells exert a net inhibitory effect. There is now direct evidence to support these hypotheses, since selective activation of Off cells has been shown to produce behavioural antinociception (Heinricher et al., 1994, Heinricher et al., 2001b), whilst selective activation of On cells has been shown to enhance nociception (Neubert et al., 2004). An amalgam of data from many studies suggests that activation of On cells is critical for the hypersensitivities associated with a range of pain states (Porreca et al., 2001, Neubert et al., 2004, Vera-Portocarrero et al., 2006). Selective On cell activation (by focal infusion of very low dose neurotensin) produces potent facilitatory effects that are sufficient enough to elicit paw withdrawal in naïve animals

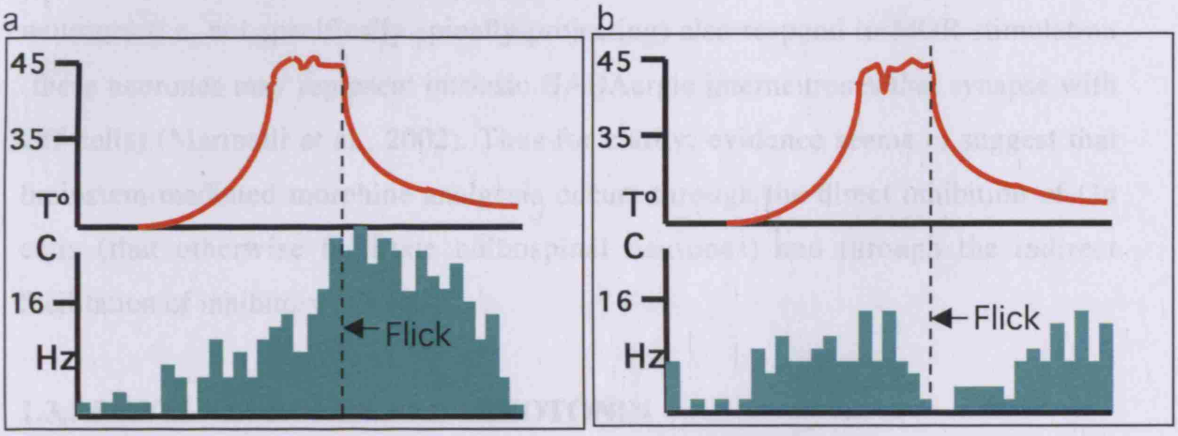
with no pre-existing sensitisation (Neubert et al., 2004). On a similar note, dipping the hindpaw in hot water has been shown to activate On cells and facilitate the heat-induced withdrawal of the contralateral limb (Morgan and Fields, 1994). Yet despite the RVM's diffuse influence and bilateral projections to multiple spinal cord segments, activation of On cells can not produce behavioural hyperalgesia in the absence of an external stimulus (for example secondary hyperalgesia induced by mustard oil application is restricted to the treated limb only despite a related increase in On cell discharge (Kincaid et al., 2005)). This therefore suggests that On cells, and the RVM in general, act in concert with the peripheral nervous system and spinal cord to alter nociceptive transmission in the dorsal horn (Urban et al., 1996).

On the basis of original RVM electrophysiological recordings, it was traditionally thought that the activities of On and Off cells were biphasic and directly out of sync. This view has since changed and it is now accepted that both groups of cells can be active at the same time, which means that the net output in the spinal cord depends on the variable and relative weights of the excitatory and inhibitory influences. Indeed, in the presence of colonic inflammation, microinjecting NMDA receptor antagonists into the RVM attenuates behavioural hypersensitivities, whilst AMPA or kainite receptor antagonists enhance hypersensitivities, which highlights that in the presence of inflammatory pain, inhibitory and facilitatory neurones are simultaneously active (Coutinho et al., 1998). In addition, it has been shown that there is an increase in both the On cell discharge and number of Off cells recorded from in awake arthritic rats (Montagne-Clavel and Oliveras, 1994) which weakens the 'mutually exclusive' concept and the associated theory that On cells function as inhibitory interneurones within the RVM, suppressing Off cell activity (this theory has been further dispelled by others who have shown that interrupting On cell activity does not disinhibit Off cell discharge (Heinricher and McGaraughty, 1998, Neubert et al., 2004)). Thus On cells may lack a global influence once activated (as alluded to above), due to the parallel engagement of Off cells that may feasibly suppress excitability in circuits serving other (uninjured) areas of the body.

Long-term plasticity in the RVM associated with chronic pain and nerve injury may be partly attributable to changes at the level of neutral cells. It is thought that these cells can switch responsiveness and become either On or Off cell-like in

certain circumstances (Urban et al., 1999). In the context of hindpaw inflammation, this allegiance is said to last for 24 hours (if not longer), a time-scale that correlates with temporal changes in RVM activity during injury. Additionally, chronic morphine exposure has been reported to increase the number of RVM On cells at the expense of neutral cells (Meng and Harasawa, 2007). It has therefore been proposed that chronic morphine sensitises a subpopulation of RVM neutral cells to noxious stimulation, which may well account for the clinical and laboratory phenomenon of opioid-induced hyperalgesia (Mao et al., 1994, Celerier et al., 2001, Ossipov et al., 2005).

Figure 1.3 *Diagram showing the typical firing responses of RVM On cells (a) and Off cells (b) just prior to nociceptive withdrawal of the tail ('flick') in response to noxious heat ~45°C. On cells accelerate firing and Off cells show a pause in activity immediately before the reflex response. (Adapted from(Heinricher et al., 1987)).*



1.3.3.4 THE RVM AND OPIOID ANALGESIA

Opioid receptor expressing areas in the PAG and RVM co-extend with known pain modulating areas (Bowker and Dilts, 1988). The RVM's central role in opioid analgesia was established following observations that anti-nociceptive doses of μ-opioid receptor (MOR) agonists in the RVM could elicit antinociception (Yaksh et al., 1976, Dickenson et al., 1979) and that inactivating this brainstem area could attenuate the anti-nociceptive actions of systemically administered morphine (Proudfit, 1980). RVM neurones express multiple opioid receptor subtypes, with functionally distinct neurones bearing different expression profiles (Marinelli et al., 2002). It has been shown in separate studies that MOR agonists directly hyperpolarise and inhibit facilitatory neurones (putative On cells), and reduce excitatory post-synaptic currents

in 52% of spinally-projecting neurones (Heinricher et al., 1992b, Marinelli et al., 2002). It has also been shown that equivalent doses of this agonist can inhibit RVM inhibitory post-synaptic currents and activate Off cells. Seeing as the direct cellular actions of morphine are mediated through a G protein coupled inhibitory receptor, and also that MOR and the GABA synthesising enzyme GAD double-label in a subset of RVM neurones, it is highly likely that morphine-induced activation of Off cells occurs via disinhibition. On the other hand it is thought that inhibitory actions on On cells occur via more direct synaptic connections. Consistent with these suggestions are findings that enkephalin-containing terminals in the RVM directly appose the axons of intracellularly labelled On cells but not Off cells (Mason et al., 1992). Furthermore, slice recordings have shown that 78% of spinally-projecting RVM neurones directly respond to MOR agonists (these responsive neurones may fully or partially represent the population of On cells) whilst 88% of 'unidentified' RVM neurones (i.e. not specifically spinally-projecting) also respond to MOR stimulation (these neurones may represent intrinsic GABAergic interneurones that synapse with Off cells) (Marinelli et al., 2002). Thus for clarity, evidence seems to suggest that brainstem-mediated morphine analgesia occurs through the direct inhibition of On cells (that otherwise facilitate bulbospinal neurones) and through the indirect facilitation of inhibitory Off cells

1.3.3.5 RVM NEURONES AND SEROTONIN

The heterogeneity of RVM neurones extends beyond their responsiveness to opioids and their ability to modulate spinal cord activity, since they also have variable electrophysiological and pharmacological properties (Mason, 1997, Marinelli et al., 2004, Zhang et al., 2006). In particular, different opioid-responding neurones stain differently for tryptophan hydroxylase (TPH), a cellular marker of serotonin (Marinelli et al., 2002). Early ideas suggested that RVM-mediated morphine analgesia occurred via the actions of serotonin (sourced from the RVM) on inhibitory 5HT receptors in the dorsal horn of the spinal cord (Wigdor and Wilcox, 1987). However, direct cellular recordings from NRM neurones have since shown that activation of serotonergic neurones is not necessary for morphine analgesia (Gao et al., 1998, Arvidsson et al., 1995). Notwithstanding observations that systemic morphine increases the concentration of 5HT metabolites in the RVM (Rivot et al., 1988, Rivot

et al., 1989), and that intrathecal 5HT receptor antagonists can modulate the consequent analgesia (Wigdor and Wilcox, 1987), it may be the case that morphine affects other RVM-driven processes, such as behavioural state, which may themselves alter serotonergic tone. Thus, a morphine-evoked increase in 5HT release may be indirect and secondary to primary opioid effects (Gao et al., 1998).

The terminals of descending serotonergic axons are well placed in superficial dorsal horn laminae to modulate nociception. The capacity of spinal 5HT to decrease or increase spinal neuronal responses is attributable to functionally opposing receptor subtypes (Yaksh and Wilson, 1979, Hylden and Wilcox, 1983). The overlap between TPH-ir and MOR-expression in spinally-projecting RVM neurones lends support to the notion that facilitatory brainstem neurones release 5HT onto excitatory receptors in the dorsal horn to enhance spinal neuronal responses (Marinelli et al., 2002, Suzuki et al., 2002b). The pro-nociceptive actions of spinal 5HT have been well described and are mediated, at least in part through fast ionic $5HT_3$ receptors. Nociceptive facilitation can be reduced by intrathecal $5HT_3$ receptor antagonists such as ondansetron (Zhuo and Gebhart, 1991, Suzuki et al., 2002b), or by depletion of endogenous spinal 5HT (Rahman et al., 2006). The strength of the inhibitions and the effects of the depletions increase after experimental neuropathy which points to an increased role of serotonergic facilitations in long-term pain states. Consistent with this, LTP-like phenomena in deep dorsal horn neurones, which correlate with increased cellular expression of the immediate early gene *zif268* (a marker of neuronal activation) in superficial laminae, have been shown to be regulated by $5HT_3$ receptor-mediated descending facilitatory controls (Rygh et al., 2006).

The exact nature of the link between serotonin neurones and On and Off cells in the RVM remains uncertain and precise correlations should not be assumed. However, discriminant analyses of 5HT and non-5HT discharge patterns *in vivo* show that serotonergic neurones have broad action potentials and low firing rates (Xu et al., 1998) which are similar to the electrophysiological properties of On cells, whilst Off cells also have immunohistochemical and action potential characteristics similar to serotonergic cells (Pan et al., 1990). It therefore seems likely, if not plausible that some, but not all On and Off cells contain serotonin, and in reverse that some but not all RVM serotonergic cells are On or Off cells (Potrebic et al., 1994). The evidence

supporting the existence of serotonergic On cells in the RVM does not however explain results from a study which showed that the majority of intracellularly labelled neurones containing 5HT in the RVM could *not* be driven by noxious heat (Mason, 1997). There may however have been a problem with the sampling in this study since only a relatively small number of RVM neurones were tested.

In addition to 5HT, noradrenaline (NA) can directly and indirectly modulate spinal sensory processing, and descending noradrenergic terminals are found in spinal loci similarly occupied by serotonergic axons and the central terminals of primary afferent fibres (Fleetwood-Walker et al., 1985, Suzuki et al., 2002a). The combined and inter-dependent actions of the monoamines on sensory signals in the dorsal horn underlie the analgesic potential of many anti-depressant agents in the treatment of neuropathic pain.

1.3.3.6 ANTI-DEPRESSANTS FOR THE TREATMENT OF NEUROPATHIC PAIN

Although central mechanisms of pain and depression utilise similar neurochemical substrates, and the diffusely organised limbic and sensory areas connect together, it is important to emphasise that anti-depressants confer analgesic actions in the absence of depressive symptoms. Empiric observations in 1977 alerted researchers to the analgesic properties of tricyclic antidepressants (TCAs) in diabetic neuropathy, and since then their efficacy has been repeatedly demonstrated in experimental and clinical settings. Their ability to modulate 5HT and NA availability, block voltage-gated Na^+ channels (Ishii and Sumi, 1992) and possibly voltage-gated Ca^{2+} channels too (Lavoie et al., 1990) means that TCAs head the therapeutic armamentarium for neuropathic pain and are often the first-line choice in the treatment of diabetic neuropathy, PHN, and post-mastectomy pain syndrome. A meta-analysis of several studies concluded that 30% of patients with neuropathic pain showed a 50% improvement in pain intensity following the use of anti-depressants (Sindrup and Jensen, 2000), which correlates with the TCAs' number needed to treat[2] (NNT) of 2-3. Serotonin and noradrenaline reuptake inhibitors (SNRIs) such as

[2] NNT is a statistical term that allows the effectiveness of different analgesics to be compared. It refers to the number of patients who need to be treated to prevent one adverse outcome. The lower the number the more effective the analgesic.

venlafaxine lag behind with a NNT of 4-5, whilst lagging still further are the selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, which have an anlagesic NNT of 7. Some of the data on SSRI efficacy controversially suggest that they do not ameliorate painful symptoms (Max et al., 1992), whilst other data report equivocal responses with efficacy described as 'moderate' at best, or 'clinically insufficient' (Saarto and Wiffen, 2005, Sindrup et al., 2005). What limited analgesic activity they possess may have more to do with their speculative ability to block voltage-gated Na^+ channels and less to do with their ability to alter 5HT tone (Deffois et al., 1996). Indeed on balance, NA mechanisms are deemed more important than 5HT mechanisms in the analgesic actions of SNRIs. This class of drugs is being forwarded as an alternative to TCAs, not due to superior efficacy (although they are effective) but instead due to superior tolerability in light of the risks and morbid side effects that are associated with higher doses of TCAs. In addition, results from a small randomised clinical trial of neuropathic pain patients recommend the use of duloxetine and possibly other SNRIs as adjuvants for gabapentin in circumstances where therapeutic benefits of the latter plateau, for example due to a ceiling of efficacy, or pharmacodynamic tolerance (Wernicke et al., 2006).

The potential synergy between duloxetine and gabapentin may be indicative of important links between brainstem areas (particularly those areas involved in 5HT and NA release) and treatment efficacy. It has been shown that the anti-hyperalgesic effects of systemic or local GBP in nerve-ligated animals can be compromised either by the depletion of central NA, or through antagonism of central α_2 receptors (Tanabe et al., 2005). The implication therefore is that GBP's effects are partly mediated by the descending noradrenergic system acting through α_2 receptors in the dorsal horn of the spinal cord. Moreover, it has also been shown that the spino-bulbo-spinal circuit, which terminates on $5HT_3$ receptors in the dorsal horn, is permissive for GBP's inhibitory actions in neuropathic pain (Suzuki et al., 2005); when neurones lying at the origin of this loop are selectively ablated, or when spinal $5HT_3$ receptors are antagonised by ondansetron, GBP fails to inhibit neuronal responses in nerve-ligated animals, whilst stimulating dorsal horn $5HT_3$ receptors in normal animals with no pre-existing pain state can induce GBP's state-dependent inhibitory actions. Hence, given that the spino-bulbo-spinal circuit and brainstem structures such as the RVM and locus coeruleus contact and receive input from affective areas such as the amygdala

and ACC, results from these separate studies propose neurochemical mechanisms by which emotions may modulate pain and its treatment. Variable activity in these areas, which may be influenced by fear, anxiety and stress, could therefore explain why patients from a seemingly uniform pain group often vary widely in their responses to analgesics such as GBP.

1.3.3.7 DESCENDING FACILITATORY INFLUENCES FROM THE RVM AND NEUROPATHIC PAIN

The barrage of peripheral input into the spinal cord after nerve injury is known to decline significantly over the course of a week despite maintained and possibly elevated pain behaviours. Persistent input and enhanced transmitter release from injured fibres drives plastic changes at spinal and supraspinal sites to elicit an increase in the RVM's facilitatory output, which thus identifies the brainstem as the source of the behavioural support and the propeller of the self-sustaining nociceptive circuit (Gardell et al., 2003). The descending facilitatory pathway from the RVM to the spinal cord in neuropathic pain states is sensitive to the excitatory effects of endogenously released cholecystokinin (CCK) acting on brainstem CCK_B receptors (Xu et al., 1993). Experimental peripheral neuropathy induces marked pronociceptive changes in the response properties of cells in the RVM, and plastic changes vary with the elapsed post-operative period (Goncalves et al., 2007). In particular, these changes include an increase in the baseline discharge rate of facilitatory On cells, and a concomitant decrease in the baseline discharge rate of inhibitory Off cells, which therefore means that both cell types promote hypersensitivities in a tonic fashion after nerve injury. However, manipulations that disrupt descending controls (for example anaesthetic block of the RVM, DLF tract lesions or selective ablation of RVM facilitatory neurones) only affect behavioural hypersensitivities after post-operative day 3 (Kovelowski et al., 2000, Burgess, 2002), which suggests that there is a time-dependency related to these supraspinal influences. In essence, the brainstem must first be informed of the CNS insult and become charged by ascending tract fibres before it can contribute towards (the maintenance of) the pain.

***Table 1.2a-b** Management of Neuropathic Pain*

1.2a First-Line Pharmacological Treatment

AGENT	EXAMPLE	NNT
VGCC $\alpha_2\delta$ binder	Gabapentin, Pregabalin	~3
Opioids	Morphine, Oxycodone	~2.5 ~2.6
Opioids with paracetamol	Co-codomol	~3.8
Atypical centrally-acting opioid & SNRI	Tramadol Hydrochloride	~2.4
Local Anaesthetic	5% Lignocaine Patch	~4.4
Tricyclic Antidepressants	Amitriptyline, Noritriptyline	~2.3 ~3

Multiple mechanisms at multiple sites may underlie neuropathic pain in a single patient, so there is an empirical and theoretical basis for recommending that patients who do not respond to one of these drugs be switched to another. In addition to sequential treatment, combination therapy can be given with first-line drugs when there is only a partial response to one of the agents. When there is not a satisfactory response to any of the first-line drugs, second- line approaches can be used.

1.2b Second- Line Pharmacological Treatment

AGENT	EXAMPLE	NNT
Anti-convulsants	Lamotrigine Carbamazepine	~2 ~3
Other Anti-depressants SNRIs SSRIs	 Venlafaxine Fluoxetine	 ~4 ~7

Drugs that may be tried when first and second line treatments do not provide satisfactory analgesia, or drugs that may be used as adjuvants include capsaicin (5% patch), clonidine, cannabinoids, clonazepam, mexiletine and NMDA receptor antagonists. Non-pharmacological treatments for neuropathic pain include neurosurgical lesions, neural blocks, functional neurosurgery, TENS, physical therapy and psychological therapy. Pharmacotherapy and/or invasive procedures must be used within a treatment context that also includes support, education and reassurance.

1.4 AIMS OF MY THESIS

The experiments described in this thesis were performed in order to clarify the contribution of the descending modulatory system to the spinal processing of sensory information in normal and pathophysiological states. The literature to date mostly describes the behavioural changes that occur following manipulation of the RVM (Pertovaara et al., 1996, Porreca et al., 2001, Neubert et al., 2004) yet relatively little is known about corresponding changes that occur in the dorsal horn of the spinal cord, a major site of sensory modulation. It was therefore my intention to use the existing data as a guide to provide a neuronal correlate for the effects seen following alteration of the brainstem's output. Moreover, the nature of *in vivo* recordings enables an examination of the modality and suprathreshold sensitivity of this control, above and beyond that enabled by examination of behavioural scores. Thus by silencing the RVM with the local anaesthetic lignocaine and measuring changes in the evoked responses of deep dorsal horn neurones to a range of peripheral stimuli, my first aim was to determine the predominant nature, facilitatory or inhibitory, of the descending drive in normal anaesthetised animals and to see which responses in particular were affected by RVM block. I then wanted to compare and contrast, both qualitatively and quantitatively, the outcomes in normal animals to the outcomes in animals with a neuropathic injury, to establish whether there are indeed enhanced facilitations of spinal neurones in the latter as the behavioural data suggests. Experimental neuropathic pain was therefore induced by spinal-nerve ligation (SNL) surgery as previously described (Kim and Chung, 1992), and was confirmed by behavioural testing paw withdrawal responses to mechanical punctate stimuli during a two-week post-operative period.

Having provided a neuronal correlate for the behavioural data, with similarities and differences in the nature of the descending control between normal and nerve-injured animals highlighted, my next aim was to characterise the pharmacology of the descending system. In particular I wanted to expand on the behavioural data concerning the pro-nociceptive consequences of CCK's actions in the RVM (Heinricher and Neubert, 2004, Xie et al., 2005). Thus, in a similar way as that described for lignocaine above, I injected CCK into the RVM and characterised changes in the evoked neuronal responses in the two groups of animals (i.e. normal

and neuropathic) to shed further light on the specific circuitry of the central CCKergic system.

Following this, I wanted to determine the contribution of facilitatory μ-opioid receptor (MOR) expressing cells in the RVM to spinal sensory processing in the normal and pathophysiological states. To do this I injected a targeted neurotoxin, dermorphin-saporin, into the RVM to ablate these cells and thereafter measured the behavioural and electrophysiological consequences of this injection relative to rats that had received a control injection. Given that a major spinal target for the descending facilitatory system is the excitatory $5HT_3$ receptor (Suzuki et al., 2002b), I further wanted to see whether, and if so how, the responses of dorsal horn neurones to spinal ondansetron, the $5HT_3$ receptor antagonist, would be affected by this ablation and consequential loss of descending facilitatory input. This particular experiment was performed to clarify the link between the brainstem's serotonergic system and the descending facilitatory system (Rahman et al., 2006).

Finally, having examined the role of RVM MOR expressing cells to the maintenance of central sensitisation and the behavioural manifestations of pain in SNL animals, I wanted to investigate the contribution of these cells to the state-dependent inhibitory effects of pregabalin, a drug used in the mainline treatment of neuropathic pain. Like gabapentin, pregabalin works, at least in part, by altering Ca^{2+} conductance at the central terminals of primary afferent neurones (Luo et al., 2001). I therefore investigated whether the state- and time-dependent actions of this drug are a function of a potential interaction between activity at presynaptic VGCCs and $5HT_3$ receptors. Given that 5HT has a key role in setting mood and emotion, this hypothesis advocates a physical inter-dependence between the mutually affecting sensory and emotional components of nociception that may influence pain scores and treatment outcome.

These experiments, which employed behaviour, *in vivo* electrophysiology, pharmacology and immunohistochemistry techniques, were undertaken to further the understanding of the complex supraspinal mechanisms that shape sensory processing at the level of the spinal cord.

2. METHODS

2.1 ANIMALS

Experiments were performed on male Sprague-Dawley rats (Central Biological Services, University College London) of varying weights (between 130g and 400g as appropriate). Rats were housed in the Biological Services Unit in cages (a maximum of 4 per cage) under a 12-hour alternating light/dark cycle with *ad libitum* access to food and water. All animal experiments were approved by the United Kingdom Home Office and were carried out in accordance with guidelines set by personal and project licenses.

2.2 STEREOTAXIC INTRA-RVM INJECTIONS OF DERM-SAP OR CONTROL COMPOUNDS

In a designated procedure room, rats weighing approximately 130g were deeply anaesthetised with ketamine (1.5mg/kg i.p) and once areflexive were secured with ear bars in a stereotaxic head-frame such that the top of their shaved and exposed cranium was completely level (equal dorso-ventral co-ordinates for bregma and lambda). A small incision was made with a dental drill at bregma –9.5mm following the midline. A Hamilton syringe was gradually driven down –9.0mm from the cranium into the RVM. The drug solution was slowly expelled and the syringe was left in position for a few minutes to minimise backflow of drug. The syringe was withdrawn and the surrounding skin sutured back together with absorbable 3-0 silk sutures. Rats recovered in an incubator before being re-housed.

2.3 SPINAL NEVE LIGATION SURGERY

Experimental neuropathic pain was established by tightly ligating the L5 and L6 spinal nerves as previously described (Kim and Chung, 1992) (Chapman et al., 1998b). Within a sterile theatre, rats were anaesthetised (1:1 O_2:N_2O, 3% halothane for induction, 1% maintenance) and placed in a prone position on a heating blanket. A section of their back fur was shaved and the underlying skin was sterilised with Betadine Antiseptic Solution (Seaton Healthcare Group, UK). A midline incision was

made with a scalpel blade, and then following a smaller left-side incision at approximately L4-S2, paraspinal muscle and fat was removed from spinous processes so that the L6 traverse process and the sacrum were visible under a microscope. Part of the L6 traverse process was clipped with rangeurs to expose the parallel-lying L4 and L5 spinal nerves. The L5 nerve was isolated and hooked with a finely-pulled glass rod and thereafter tightly tied with non-absorbable 6-0 silk thread distal to the dorsal root ganglion (and proximal to the formation of the sciatic nerve). The L6 nerve was then hooked from under the sacrum and tied in a similar way. Haemostasis was confirmed and the wound was sutured with 3-0 absorbable silk. The surrounding skin was pulled together and secured over the injury with wound clips. Rats recovered in an incubator, and once it had been confirmed that they had no observable motor impairment in their left hindpaw, were re-housed in cages as above.

2.4 ASSESSMENT OF BEHAVIOURAL HYPERSENSITIVITIES FOLLOWING SNL SURGERY

After SNL surgery, rats typically displayed good ongoing health, weight-gain, grooming and activity levels, and showed no overt signs of distress. On post-operative days 2, 7, 9 and 14 (unless otherwise stated), behavioural signs of punctate mechanical and cooling hypersensitivity were assessed in the left ipsilateral hindpaw relative to the right contralateral hindpaw in awake and alert rats. Rats were gently handled and individually placed in clear acrylic cubes on an elevated floor of wire mesh. Following a period of acclimation (30 minutes), mechanical sensory thresholds were determined by paw withdrawal to vF 1g, 5g, 9g and 15g (tested in consecutive, ascending force). Each filament was applied to the plantar surface of the paw for ~2-3s with enough force to cause buckling, and for each animal this was repeated 10 times at set positions on each paw. The number of lifts in response to each of the filaments was noted for each paw and expressed as a percentage response. Cold hypersensitivity was assessed by applying a drop of acetone to each paw (5 times during each testing period, with each application separated by an interval of at least 5 minutes), and as above, the number of lifts was expressed as a percentage response.

2.5 *IN VIVO* ELECTROPHYSIOLOGY

2.5.1 ANAESTHESIA AND EXPERIMENTAL SET-UP

Experiments were performed in the laboratory as previously described (Dickenson and Sullivan, 1986) Anaesthesia was induced in a chamber using 3% isoflurane (Baxter International Inc.) in 66% N_2O and 33% O_2, and was thereafter maintained by tracheal intubation (with a polyethylene tube of tip diameter 80-100mm) with the isoflurane level set at 0.7%. Areflexive rats were then secured centrally in a stereotaxic frame by ear bars, and vertebral clamps were placed rostral and caudal to allow a laminectomy to be performed whereby the posterior arches of vertebrae L1 – L3 were clipped to expose L4 – L5 segments of the underlying spinal cord. Core body temperature was monitored and maintained at 37°C by a homoeothermic heating blanket unit and rectal probe (Harvaard Homoeothermic Blanket Control Unit).

2.5.2 NEURONE ISOLATION

A parylene-coated tungsten electrode (125mm diameter, A-M Systems, WA, USA) placed in a head-stage was moved laterally and rostral-caudally outside the spinal cord, and dorsoventrally within the spinal cord using a manual micromanipulator that could make gross and fine movements. Neurones receiving afferent A-fibre and C-fibre input from the hindpaw were sought by periodic light tapping, and occasionally pinch of the glaborous surface of the hindpaw.

2.5.3 TESTING PROTOCOL: MEASURING EVOKED NEURONAL RESPONSES

Following the isolation of a neurone (i.e. a prominent single responsive neurone that could be counted in the absence of background activity and extraneous cells) in the deeper laminae of the dorsal horn (approximately 500 - 1000μm from the surface of the cord), it's A- and C-fibre thresholds were tested by placing electrical pins connected to a NeuroLog stimulator (Digitimer, UK) transcutaneously into the centre of the cell's receptive field. All neurones used in these studies had defined

receptive fields in the toe region of the hindpaw and responded to both electrical and natural stimuli. Once its thresholds were ensured to be within the range of 0.1 – 3.3mA, a train of 16 electrical stimuli were delivered to the centre of the receptive field at three times the threshold current for C-fibre activation, with a stimulus frequency of 0.5Hz (2ms pulse width). Neuronal responses to peripheral stimulation were captured, amplified, filtered and discriminated by a CED micro1401 interface (Cambridge Electronic Designs, UK), visualised on a connected digital storage oscilloscope and were used to construct post-stimulus-time histograms on a Pentium computer monitor using Spike 2 software.

Responses evoked by Aβ-, Aδ-, and C-fibres were separated according to latency (0-20ms, 20-90ms and 90-350ms respectively) on the basis that different fibre types propagate action potentials at different conduction velocities (see Section 1.3.1.1). This therefore means that following spike generation in the periphery, action potentials conducted along Aβ-fibres reach the synapse and excite 2nd order dorsal horn neurones faster than action potentials conducted along Aδ-fibres, which themselves reach the spinal cord more readily than action potentials conducted along C-fibres, hence the time-dependent order of separation. Action potentials occurring after 350ms are noted down as post-discharge. These parameters are specific for adult rats weighing approximately between 250g – 300g, thus in the event of experiments performed on rats weighing either more or less than this, the parameters were simply changed on the Spike 2 script to reflect the altered distance from periphery to spinal cord. The number of action potentials triggered by the first of the 16 electrical stimuli, referred to as 'input' (a measure of the non-potentiated response) were recorded, as was 'wind up' – the increase in neuronal responsiveness following repeated stimulation at the same intensity, calculated as the total action potentials evoked after 16 stimuli minus '16 x the input'.

The receptive field was also stimulated by a range of innocuous and noxious natural stimuli for 10 seconds each. These stimuli included brush, von Frey filaments with bending forces of 1g, 6g, 8g, 15g, 26g and 60g (Scientific Marketing Associates, UK) as well as temperatures ranging from 35°C - 50°C. Thermal stimuli were applied with a constant water jet onto the centre of the receptive field. Single unit activity was captured and analysed as described above, and was displayed on the computer in the

form of a ratemeter. Stabilisation of a neurone's responses was confirmed with at least 3 consistent responses (<10%) to all measures. For characterisation studies, means of the baseline responses (as above, at least 3 per stimulus) were calculated. For pharmacological studies, mean scores calculated for each of the pre-drug evoked responses were compared with post-drug values (at set intervals after drug administration).

2.6 ANIMAL PERFUSION, BRAINSTEM REMOVAL & TISSUE STORAGE

After *in vivo* electrophysiology experiments it was sometimes necessary to perfuse the rat and remove brainstem tissue either for verification of RVM injection site or immunohistochemistry (when these procedures were not required, rats were killed with an overdose of isoflurane at the end of the experiment). Anaesthetised rats were perfused intracardially with physiological saline followed by 10% paraformaldehyde using a peristaltic pump, and the brainstem was removed, post-fixed in PFA and cryo-protected in 30% sucrose. 40mm transverse sections were then cut on a freezing microtome and serially collected in wells filled with 0.1M PBS.

2.7 HISTOLOGY

Drugs that were injected into the RVM during the electrophysiology experiment were mixed with a very small drop of pontamine sky blue so that upon removal and examination of the brainstem, injection site could be verified under a microscope. These sites were subsequently plotted onto corresponding representative sections from the rat brain atlas centred around the RVM at bregma –9.84 (Paxinos and Watson, 2005)

2.8 IMMUNOHISTOCHEMISTRY AGAINST THE MU OPIOID RECEPTOR

Free-floating brainstem sections were washed with 0.1M phosphate buffered saline (PBS) and blocked with normal goat serum (Vector, Peterborough, UK) for 30 minutes. Sections were then incubated on a rocker overnight at room temperature with

polyclonal rabbit μ-opioid receptor RA10104 antibodies (Neuromics, Minneapolois, USA) in PBS containing 2% normal goat serum and 0.4% Triton (Sigma, St. Louis, USA). (N.B. The primary antibody, which for the purpose of these reactions was diluted by a factor of 20,000, was re-constituted by adding 1ml distilled water and stabilised with immunohistochemical grade bovine serum albumin. This primary antibody was used as it has been previously characterised and validated against the μ-opioid receptor (Nandi et al., 2004). Controls were incorporated into the procedure by testing primary antibody binding to mu-opioid expressing cells in spinal cord tissue from normal animals). After several washes, brainstem sections were incubated for one hour with biotinylated anti-rabbit secondary antibodies (Vector Laboratories, CA, USA). MOR-labelled cells were subsequently revealed using avidin-binding peroxidase complex (ABC Elite; Vectastain©, Vector Laboratories, CA, USA). Thereafter, indirect amplification was carried out using Tyramine Specific Amplification, with FITC Fluorescein Avidin D for visualisation of immunoreactive sites (Vector Laboratories, CA, USA). Sections were finally washed with 0.1M PBS and coverslipped using Vectashiled (Vector Laboratories, CA, USA), and air-dried. Images were captured on a Leica DMRE fluorescent microscope with a TCS SP1 confocal head and an oil immersion objective.

2.9 PHARMACOLOGY - AGENTS USED, SOURCE, PREPARATION, STORAGE & ADMINISTRATION

Lignocaine Hydrochloride was obtained from Hameln Pharmaceuticals, USA. Stored in ampoules at 4°C. 0.8µl of 2% (w/w) (=16ng of lignocaine) was injected directly into the RVM during electrophysiological testing.

CCK8 Sulphate was obtained from American Peptide Company, CA. The peptide was dissolved in distilled water and stored in separate aliquots at -20°C. 50ng was injected directly into the RVM during electrophysiological testing.

Dermorphin-Saporin was obtained from Advanced Targeting Systems, CA. The toxin was dissolved in distilled water and stored in separate aliquots at -20°C. 3pmol was injected into the RVM typically 4 weeks before electrophysiology testing.

Saporin as above for dermorphin-saporin.

Ondansetron (Zofran®) was obtained from GlaxoSmithKline, UK. Dissolved in 0.9% saline and stored at 4°C. 100µg/50µl was administered to the exposed surface of the spinal cord during electrophysiology.

2-methyl5HT was obtained from Sigma, UK. Dissolved in 0.9% saline and stored at 4°C. 0.1µg/50µl was administered to the exposed surface of the spinal cord during electrophysiological testing.

Pregabalin was a gift from Pfizer, UK. Stock solution was prepared in 0.9% saline and stored at 4°C, 10mg/kg was introduced systemically during electrophysiology.

Morphine Sulphate was obtained from Evans Medical Ltd., UK. Stock solution was prepared in 0.9% saline and stored at 4°C, 0.25µg/50µl and 1µ/50µl were administered intrathecally during electrophysiological testing.

Naloxone Hydrochloride was obtained from Sigma-Aldrich Company Ltd., UK. A stock solution was prepared in 0.9% saline and stored at 4°C, 50mg/50µl was

administered to the eposed surface of the spinal cord during electrophysiological testing.

2.10 DATA & STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 4 for Apple Macintosh OS 10.4, (GraphPad Software, USA), and for all data a 95% confidence interval was used as a measure of statistical significance.

Individual analyses for particular experiments are described in each chapter.

3. BEHAVIOURAL AND ELECTROPHYSIOLOGICAL CHARACTERISATION OF THE SPINAL NERVE LIGATION (SNL) MODEL OF NEUROPATHY

3.1 MERITS AND MINOR DISADVANTAGES OF THE SNL MODEL

A number of animal models have been developed in the past two decades with the express purpose of investigating both the peripheral and central mechanisms of neuropathic pain. There are various models of peripheral mononeuropathy (see Section 1.2.4 and Table 1.1), yet the SNL model in which the L5 and L6 spinal nerves are unilaterally ligated close to their respective ganglia to produce a restricted partial denervation of the hindlimb, is favoured by many for various reasons including the stereotypical injury that is induced, which gives rise to consistent and reproducible sensory abnormalities over a sustained post-operative period. The sensory abnormalities manifest as evoked (and arguably ongoing) behavioural responses that can be evaluated by sensory testing using mechanical, thermal and cooling stimuli. In head-to-head comparisons of different animal models of experimental neuropathic pain carried out by single laboratories using a standardised testing procedure, the SNL model showed the largest and most stable magnitudes of behavioural hypersensitivities to applied peripheral stimuli (Kim et al., 1997, Dowdall et al., 2005). Given that our laboratory and the studies in this thesis combine behavioural and electrophysiological techniques to look within an intact system at the evoked responses of the hindpaw and single cells to various peripheral stimuli, it is important that these responses show minimal variability between animals. The uniform pathophysiology associated with the SNL model is largely attributable to the requirements for tight ligatures of a specific set of nerves as opposed to loose or partial ligatures of various components of the sciatic nerve.

Perhaps the most advantageous aspect of this model however relates to the segregation of injured and intact afferents into different spinal segments (see Figure 3.1); thus the somatic contribution of the sciatic nerve is divided into injured L5 and L6 afferents, and uninjured L4 and L3 afferents (in addition to the sciatic nerve, the foot is also innervated by the saphenous nerve, and at least some of the afferent input in this nerve travels along L3 segments to the dorsal horn of the spinal cord (Baron et

al., 1988)). This neat separation means that the relative contributions of injured and intact primary afferents to the pain phenotype can be selectively studied. Indeed, this model has been used to explore the contributions of injured and uninjured primary afferent fibres to the various behavioural expressions of neuropathic pain; transection or anaesthetic block of L5/6 segments five days after SNL surgery abolished mechanical and cooling hypersensitivities and ongoing pain[3], whilst the same manipulations of intact L3/4 segments affected only evoked pain scores (Yoon et al., 1996). Another study by a different group also looked at the role of L4 afferents in the development of neuropathic pain following ligation of L5/6 spinal nerves (Lee et al., 2003). They showed that intermittent mechanical stimulation of the hindpaw ipsilateral to the side of nerve injury during the first week after SNL (which would increase activity in the intact L4 spinal afferents innervating this territory) significantly potentiated the development of mechanical hypersensitivities in the affected paw. Similarly, mild irritation of the L4 spinal nerve by gentle and repeated stretching after L5/6 ligation resulted in more severe behavioural hypersensitivities relative to unstretched controls. This stretching, caused by hooking a glass rod under the exposed nerve and sliding it back and forth across the shaft 20 times, is thought to cause an acute and possibly prolonged increase in L4 afferent drive, yet it does not affect mechanical sensitivities in the absence of L5/6 ligations (Wallerian degeneration of L5 axons after SNL may prime the uninjured nerve). The preservation of the L4 spinal nerve during SNL surgery is therefore important, and as such it must be interfered with as little as possible. Given that the L4 and L5 spinal nerves lie in extremely close proximity (see figs 3.1 & 3.2) this requires a good level of technical skill, presenting an important caveat and potential source of variability associated with this model. However, this variability is much less that that caused by unclear definitions of 'loose' and 'partial' ligations in the Bennett and Seltzer models.

The capacity to tease out injured and uninjured afferents in the SNL model of neuropathic pain allows *in vivo* recordings to be made from damaged nerves alone.

[3]Ongoing pain was surmised from specific behavioural tests: rats were acclimatised on brass plates of neutral temperatures (~30°C) or cold temperatures (~5°C) before they were assessed over the course of 5 minutes for the accumulative duration of time that their ipsilateral paw was lifted off of the plate (foot lifts associated with locomotion or body repositioning were not counted). Significant foot lifts on the neutral plate were taken as an index of spontaneous pain, whilst foot lifts on the cold plate were interpreted as cold-stress induced ongoing pain Choi, Y., Yoon, Y. W., Na, H. S., Kim, S. H. and Chung, J. M., 1994. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. Pain. 59, 369-376.

This technique, like microneurography in human neuropathic patients (see Section 1.2.3) has identified ectopic activity emanating from injured peripheral nerves, the onset of which directly correlates with the expression of behavioural hypersensitivities in awake nerve-ligated animals (Sun et al., 2005). Interestingly, these discharges, which assume tonic and bursting firing patterns within the first 24 hours of injury, gradually decline over the course of the next 14 days until they fire in an irregular and relatively quiescent fashion. This latent level of activity is not substantial enough to support the tactile hypersensitivities that can persist at a stable and significant level for up to four months (Choi et al., 1994). Thus, it has been suggested from these and similar studies in SNL animals, that ectopic discharges from damaged nerves are crucial for neuropathic pain in its early stage, but their importance wanes over time, emphasising a possible distinction between mechanisms that initiate neuropathic pain, and mechanisms that maintain the pain (as discussed in Section 1.3.2.5). The early ectopic activity is thought to trigger spinal sensitisation, which consequently amplifies input from residual intact fibres to support ongoing behavioural expressions of the injury. Certainly, on days 7-14 after SNL surgery in rats, C-fibre-evoked field potentials in the dorsal horn tend to have higher amplitudes than normal, whilst the threshold for C-fibres to initiate LTP significantly decreases, pointing once again towards a newly sensitised spinal cord after nerve injury (Xing et al., 2003).

In addition to the above mentioned changes in electrical properties of damaged nerves, single fibre recordings from L4 dorsal roots after L5/6 ligation surgery have identified a novel 'modified rapidly adapting mechanoreceptor' innervating the partially deafferented foot (Na et al., 1993). Synergism between ectopic activity in axotomised neurones and altered transduction in intact neurones may explain the hypersensitive phenomena that accompany many neuropathies, as well as spontaneous pain. Spontaneous pain characterises most human neuropathic pain states, yet the absence of verbal descriptors makes it difficult to verify, monitor and measure in corresponding animal models, with inferences from behavioural observations (see footnote above) thought to be tentative at best. As such, there have been few studies that have attempted to characterise the magnitude, time-course and pharmacological sensitivity of spontaneous pain (Choi et al., 1994, Yoon et al., 1996, Suzuki and Dickenson, 2006). Autotomy – self-injurious behaviour in which a body

part, usually denervated or deafferented, is compulsively licked and bitten – is thought by some to represent ongoing and spontaneous pain (Liu et al., 2001, Minert et al., 2007). In the SNL model, extreme autotomy (i.e. visible mutilation of the hindpaw) is not observed, instead there is just occasional licking and chewing of the affected paw and digits during behavioural testing (and high levels of spontaneous firing in deep dorsal horn neurones recorded *in vivo* (Suzuki and Dickenson, 2006)) . If extreme autotomy was taken as a corollary of extreme spontaneous pain, then some might say that the SNL model does not accurately model the human pain condition. However, the neural substrate for spontaneous pain could be present but not expressed as autotomy for good reason; in other models of peripheral neuropathic pain such as the CCI model, the hindlimb is totally insensate which means that ongoing chewing and biting would not exacerbate any potential pain felt. In the SNL model however, the intact L4 spinal nerve spares some sensory input to the hindpaw which means that the very act of autotomy would not only result in pain, but exaggerated pain given the sensitised state of the paw. In this model therefore, autotomy would be suppressed due to the presence of sensory cover, hence its absence is not necessarily an indicator of absent spontaneous pain. Arguably, the SNL model of neuropathic pain is more ethically sound than other models since partial denervation maintains some sensory control allowing the animal to guard its paw to minimise and escape cutaneous stimulation (but in a circular argument, it is not possible to accurately assess the relative levels of spontaneous pain between the models so this reasoning may be parried with criticism).

Whether or not paw guarding is an indicator of ongoing pain, paraesthesisa, dysaesthesia or some motor abnormality, it is sensitive to morphine and TCAs (Jazat and Guilbaud, 1991, Ardid and Guilbaud, 1992), and is highly reminiscent of the protective behaviour seen in human neuropathic patients who may go to extreme measures to shield their affected limb (or other body part) from even slight stimulation. In patients with causalgia (a condition first described in 1872 that is characterised by intense 'burning and darting' pain in the area served by a damaged nerve), gentle, normally innocuous stimuli applied to the skin can reportedly trigger intense pain. This allodynia is akin to the exaggerated responses that SNL animals show to brush strokes and low-threshold punctate mechanical stimuli during the post-operative period (paw withdrawal is seen in response to stimulation by vF 1g despite

nociceptors having a far greater threshold for activation (Leem et al., 1993b)). Another important feature that links human causalgic pain with the behavioural phenotype seen in SNL rats is the role of the sympathetic nervous system; both are sympathetically maintained (immunohistochemical staining has identified sprouting of sympathetic fibres into the DRGs of injured segments within 2 days of SNL surgery, with the amount of sprouting said to correlate with behavioural hypersensitivities (Kim et al., 1993), hence inactivating sympathetic components by surgery or guanethidine can relieve pain in human patients (Hannington-Kiff, 1974) (although controversial) and alleviate mechanical and cooling hypersensitivities in SNL animals (Kim et al., 1997). Given that the behavioural effects of sympathectomy were greatest in SNL animals relative to those animals that had received partial sciatic nerve and chronic constriction injuries (Kim et al., 1997), it could be said that this model most closely resembles the human neuropathic condition and thus has the best face validity.

The SNL model of neuropathic pain has also been validated pharmacologically, hence it is said to have a high degree of predictive validity. Accordingly, behavioural responses in this model are sensitive to compounds with known analgesic and anti-allodynic properties from several different drug classes, including opioids, $\alpha_2\delta$ ligands and anti-depressants (LaBuda and Little, 2005). Moreover, doses of gabapentin and morphine that are known to reverse pain-related behavioural hypersensitivities can also abolish spontaneous firing of deep dorsal horn neurones in SNL animals (Suzuki and Dickenson, 2006). These two agents have a pre-dominantly pre-synaptic site of action in the spinal cord, thus the results support the notion that ectopic activity in primary afferent fibres promotes hyperexcitability in the spinal cord, characterised not only by increased spontaneous activity, but by lowered activation thresholds to peripheral input and expanded receptive fields (Suzuki and Dickenson, 2000). In addition, the susceptibility of behavioural hypersensitivities and spontaneous neuronal firing to the inhibitory effects of gabapentin and morphine demonstrated by this model, hints once again at the early causal relationship between ectopic activity and behavioural manifestations of pain (Sheen and Chung, 1993, Xiao and Bennett, 1995, Sukhotinsky et al., 2004).

The availability of a range of highly developed models of neuropathic pain provide opportunities to study far-reaching multiple mechanisms and common underlying features of this pain state, yet adaptation of the SNL model to other animal species such as mice (which allows transgenic studies to identify the contribution of particular genes to the pain phenotype, such as that encoding the $Na_v1.8$ channel (Gold et al., 2003)) and primates (Palecek et al., 1992), as well as it's pre-clinical validity, means that this model in particular is extensively used for various investigative works on neuropathic pain mechanisms, as well as being used to identify novel targets and screen potentially analgesic agents.

3.2 METHODS

3.2.1 SNL SURGERY

Spinal nerve ligation was carried out as described in Section 2.3. Briefly, under gaseous anaesthesia a longitudinal incision was made at the lower lumbar/upper scaral level to expose the left paraspinal muscles. These were teased apart from the L5 spinous processes (see Figure 3.2) so that the L6 transverse process was clearly visible under a microscope. This was minimally clipped with small rangeurs to reveal the underlying L4 and L5 spinal nerves. The L5 nerve was accessed with a smooth glass rod and tightly ligated with a small length of non-absorbable 6-0 silk thread. The L6 spinal nerve was then hooked from under the sacrum and similarly ligated. Once haemostasis was confirmed, muscles were sutured in layers using 3-0 absorbable silk threads and the skin joined together over the injury with wound clips.

3.2.2 BEHAVIOURAL ANALYSIS

As described in section 2.4, behavioural outcomes were subsequently assessed on post-operative days 2, 7, 9 and 14 unless otherwise stated. Animals displaying motor deficits deemed abnormal for this surgery[4] were not included in the behavioural analysis or subsequent electrophysiological characterisation as it invariably indicated damage or irritation of the L4 spinal nerve (this nerve innervates many proximal muscles of the hindpaw thus its damage manifests as dragging and paralysis of the ipsilateral limb). The end-point for behavioural assessment was paw withdrawal to the applied mechanical and cooling stimuli, with responses quantified as a percentage withdrawal to the total number of applied stimuli in each category. On post-operative days 14, 15, 16 or 17, when behavioural hypersensitivities had plateaud (see Figure 3.4), *in vivo* electrophysiological responses of dorsal horn neurones in the lumbar region of the spinal cord ipsilateral to the side of nerve injury (therefore receiving afferent input from the injured paw) were recorded in response to electrical and mechanical stimulation of their peripheral receptive fields (see Section 2.5). Since the testing paradigm was minimally invasive and did not alter the pharmacology of the

[4] Normal motor deficits associated with SNL surgery include a mild inversion or protection of the foot with slightly ventroflexed toes.

system, more than one cell characterisation was made per animal. At the end of the testing day, animals were sacrificed with an overdose of anaesthetic.

3.2.3 *IN VIVO* ELECTROPHYSIOLOGY

As described in Section 2.5, however, in SNL animals all recordings were made ipsilateral to the side of nerve injury

3.2.4 DATA ANALYSIS

Behavioural responses in the SNL animals are presented as mean frequency of PWD response±SEM. Statistical significance between the responses of the ipsilateral and contralateral paws was tested using non-parametric Wilcoxon matched pairs tests.

Raw electrophysiological data are presented as evoked responses (i.e. number of action potentials)±SEM. For comparisons between the two groups, student's *t*-tests were employed for the electrical data whilst two-way ANOVA with Bonferroni corrections were used for natural data to safeguard against multiple comparisons of statistical significance on the same data. The level of statistical significance was taken as $P<0.05$.

Figure 3.1 *Schematic representation of the surgical ligation procedure used in this study showing the distribution of axotomised and non-axotomised DRG neurones. (N.B. The diagram is not strictly accurate since not all fibres in the L4 and L5 segments join the sciatic nerve).*

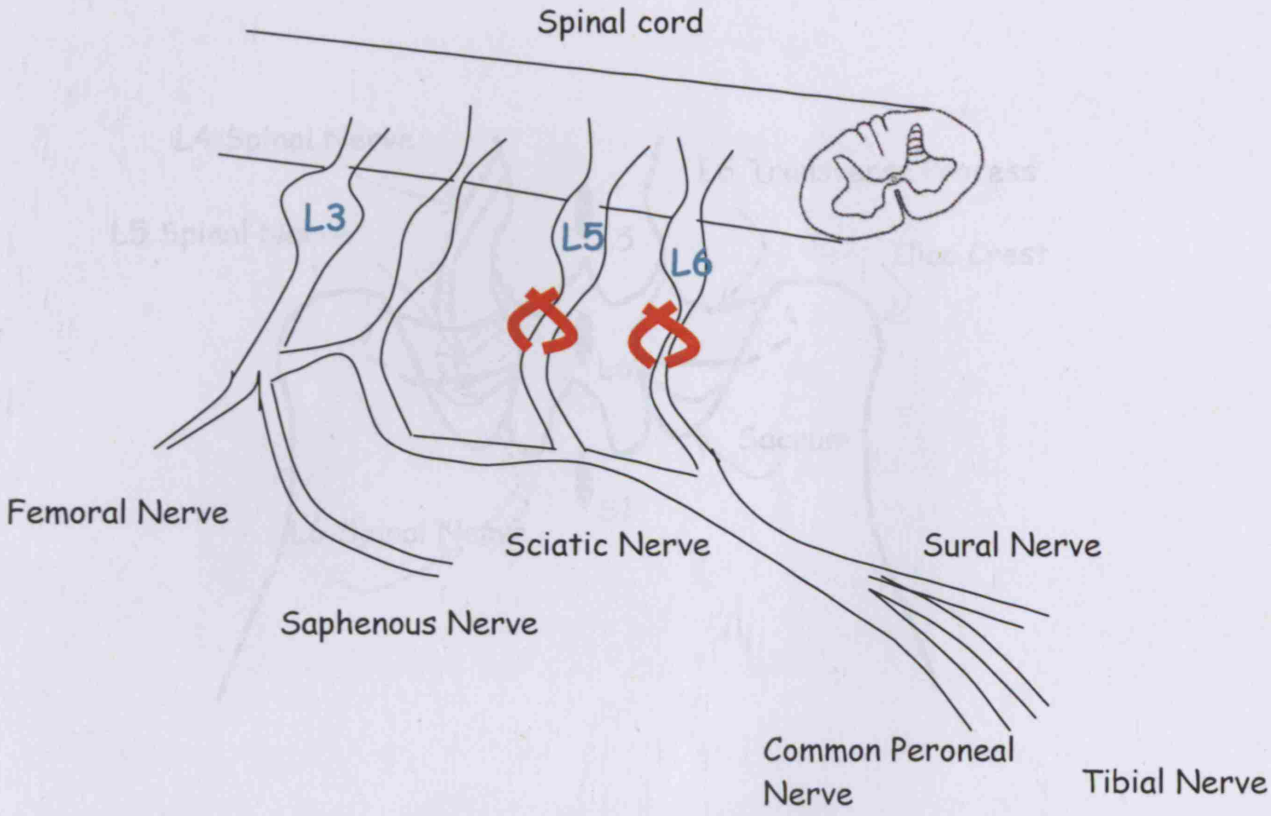


Figure 3.2 *Diagram showing the dorsal view of the bony structures and spinal nerves at the lumbro-scaral level after removal of paraspinal muscles, as seen under a microscope during SNL surgery.*

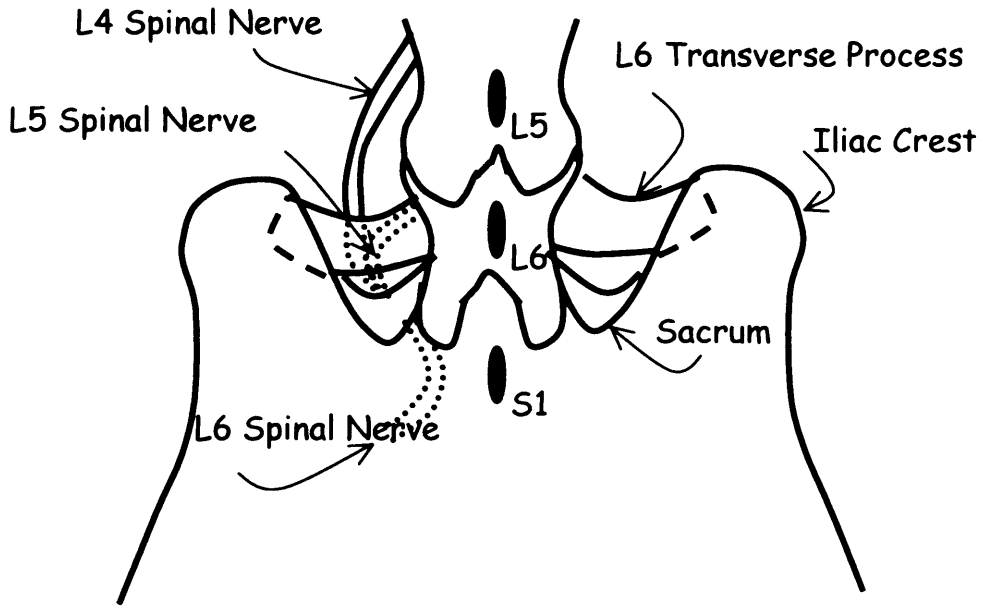
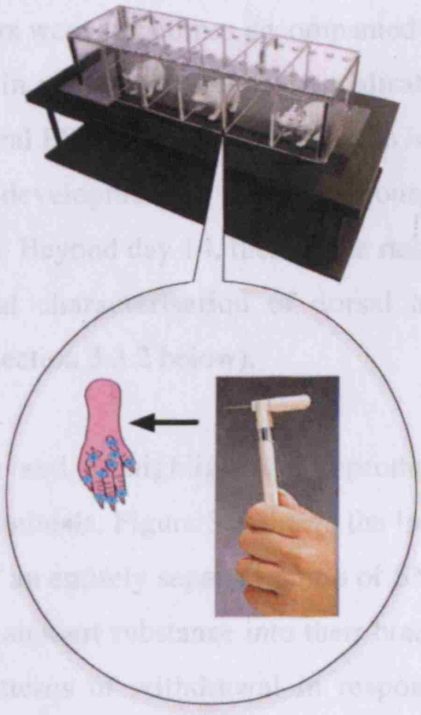


Figure 3.3 *A photograph and diagram showing the behavioural testing of a rat using a von Frey filament. Each filament was applied with enough force to cause buckling, and for each filament this was repeated ten times on each paw – twice on each of the five toes. This is known to be the most sensitive area of the injured hindpaw after SNL surgery. This is a logical approach since in nerve-injured humans with neuropathic pain, QST is carried out in areas associated with the most pain.*



3.3 RESULTS

3.3.1 BEHAVIOURAL STUDIES

For characterisation of post-operative behavioural responses in SNL animals, data from 39 operated animals were collated and mean withdrawal frequencies calculated in response to each of the stimuli on each of the testing days. Rats included in these analyses all continued in good health after the ligation surgery, displaying normal weight gain and grooming behaviours, with no notable outward signs of anxiety or aggression. Some rats guarded the affected limb as described in Section 3.1, and tended to bear more weight on their contralateral side.

Following a 30-minute period of acclimatisation, PWD frequencies were determined in response to von Freys 1g, 5g, 9g and 15g as well as acetone. As figure 3.4 shows, in both the ipsilateral and contralateral paws, PWD frequency increased linearly with the intensity of the stimulus. For each stimulus used on each of the testing days, the ipsilateral paw showed a significantly greater PWD frequency relative to the contralateral paw. Hence these results show a rapid and significant onset of mechanical and cooling hypersensitivity in the nerve-injured paw. These hypersensitive behaviours were sometimes accompanied by brisk shaking, licking and biting of the limb, and in rare circumstances, vocalisation (casual observations, no numerical data). Ipsilateral PWD frequencies tended to increase between days 7 and 9 (indicating an ongoing development of pain behaviours) before plateauing out at a constant level by day 14. Beyond day 14, these same rats were anaesthetised and used for electrophysiological characterisation of dorsal horn neuronal responses to peripheral stimuli (see Section 3.3.2 below).

For comparison and to highlight the reproducibility of the behavioural responses seen in SNL animals, Figure 3.5 shows the ipsilateral (a) and contralateral (b) PWD frequencies of an entirely separate group of SNL rats (that incidentally had received an injection of an inert substance into their brainstems, as discussed later on in this thesis). The patterns of withdrawal in response to the same stimuli are reasonably consistent for both the ipsilateral and contralateral paws between the two groups of animals, although error bars are bigger in Figure 3.5 (due to a smaller

number of animals being included in the study, n=21). The error bars associated with the responses indicate that there was a small range of PWD frequencies for each stimulus within each group of tested animals. Sources of this non-significant variation amongst similarly operated animals shall be discussed in Section 3.4 below.

Figure 3.4 *Graphs showing the withdrawal frequencies to a range of mechanical stimuli and acetone in the ipsilateral hindpaw (a) and the contralateral hindpaw (b) on various days following SNL surgery. By post-operative day 2, rats were showing signs of hypersensitivity in their injured paw, which developed progressively and remained significant during the course of the two testing weeks. Data are presented as the mean response± SEM. Significance is measured and denoted with an asterisk with respect to PWD frequencies to the same stimuli in the contralateral paw.*

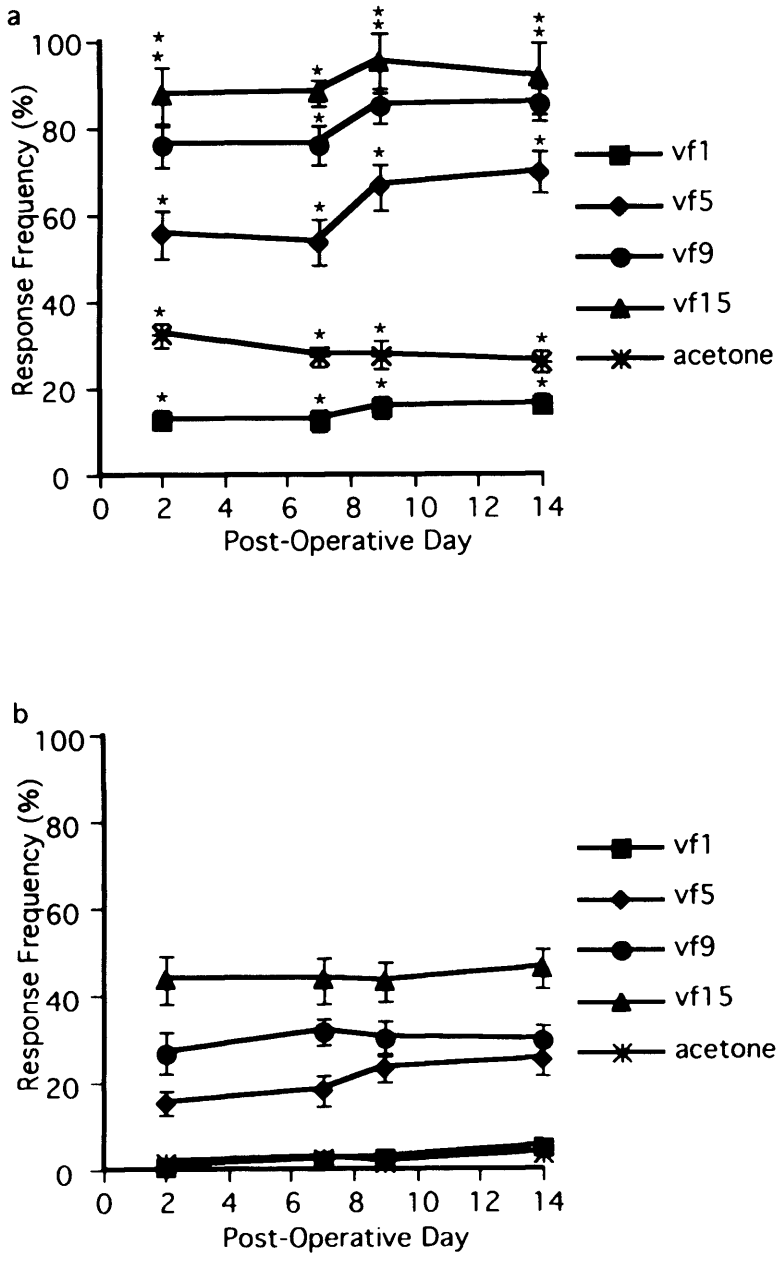
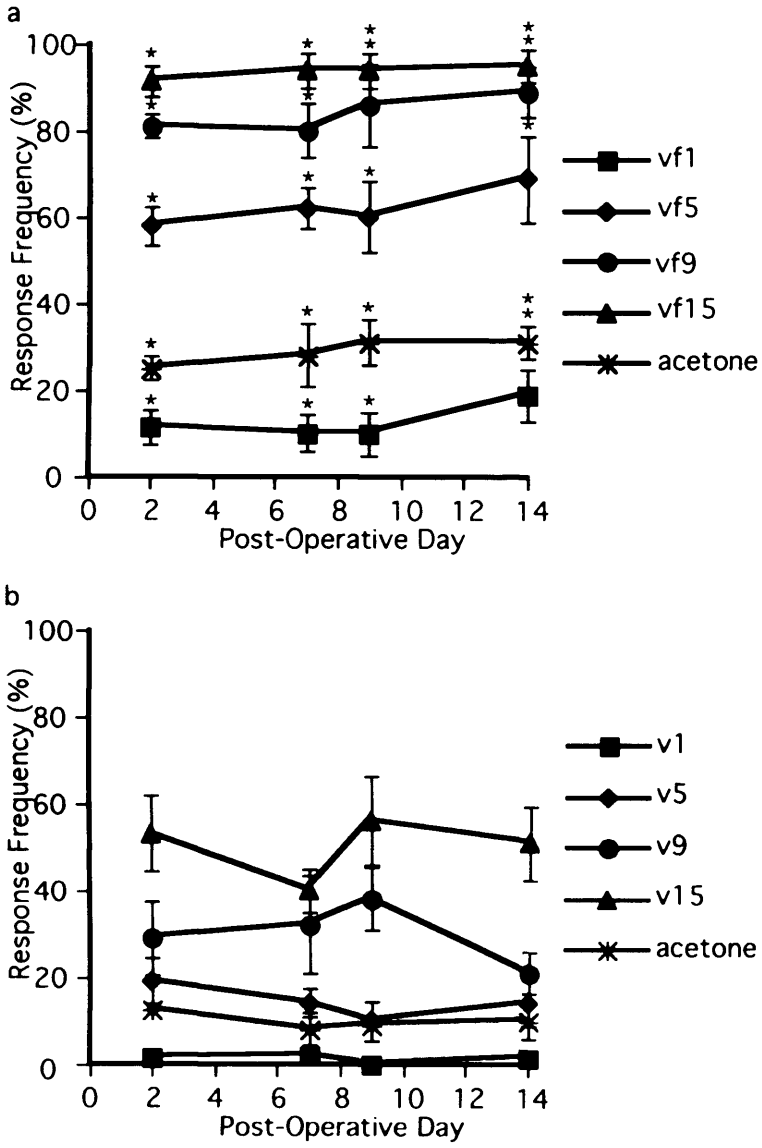


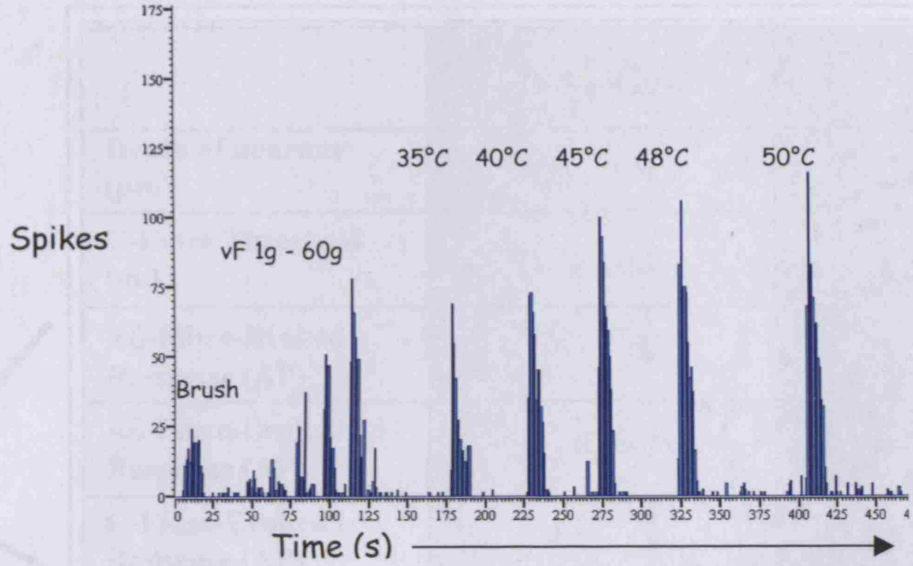
Figure 3.5 *Graphs included for comparison with Figure 3.4 showing the ipsilateral (a) and contralateral (b) PWD frequencies of a separate group of SNL rats, in response to the same stimuli on the same post-operative testing days. Data are presented as the mean response± SEM. Significance is measured and denoted with an asterisk with respect to PWD frequencies to the same stimuli in the contralateral paw.*



3.3.2 *IN VIVO* ELECTROPHYSIOLOGICAL CHARACTERISATION OF DORSAL HORN NEURONAL RESPONSES

Dorsal horn neuronal responses to peripheral stimuli were characterised and compared between SNL rats (on post-operative days 14-17) and naïve rats of similar weight. A total number of 23 SNL rats were included in this study, yielding 66 isolated neurones in the L4-L5 region of the spinal cord (N.B. all neurones recorded from in SNL rats lay ipsilateral to the side of nerve injury). In naïve rats, these numbers were 25 and 52 respectively. Neurones recorded from were located in deep laminae (>500μm from the dorsal surface of the spinal cord) and responded to electrical stimulation of their peripheral receptive fields. This was delivered transcutaneously at three times the threshold for C-fibre stimulation (never greater than 9.9mA, hence C-fibres had to have thresholds ≤3.3mA), which also recruited Aβ- and Aδ-fibres. Neurones recorded from also responded to a range of innocuous and noxious mechanical punctate stimuli (vF1g – 60g, although not all neurones responded to 1g; in naïve rats the response rate was 77% whilst in SNL rats 61% responded to this low-threshold stimulus), thermal stimuli delivered by a constant water jet (35°C – 50°C) and brush (dynamic mechanical stimulation). Figure 3.6 shows a typical ratemeter of the number of action potentials evoked by a single neurone in response to a 10s stimulation of each stimuli used (applied to the centre of the cell's receptive field).

Figure 3.6 *A typical rate recording showing the coding responses of a deep dorsal horn neurone to a range of mechanical and thermal stimuli, applied for a period of 10 seconds each.*



As Table 3.1 shows below, the depth of the neurone recorded from was not significantly different between the two populations of rats, nor was the threshold for C-fibre stimulation. Moreover, Table 3.1, and Figure 3.8a shows that there were no significant differences in the magnitudes of Aβ- or C-fibre evoked responses, input, post-discharge and wind-up between the two groups. Interestingly however, a significant difference between the Aδ-fibre-evoked responses between the two groups of rats was calculated (P = 0.025), with responses being notably greater in the SNL rats.

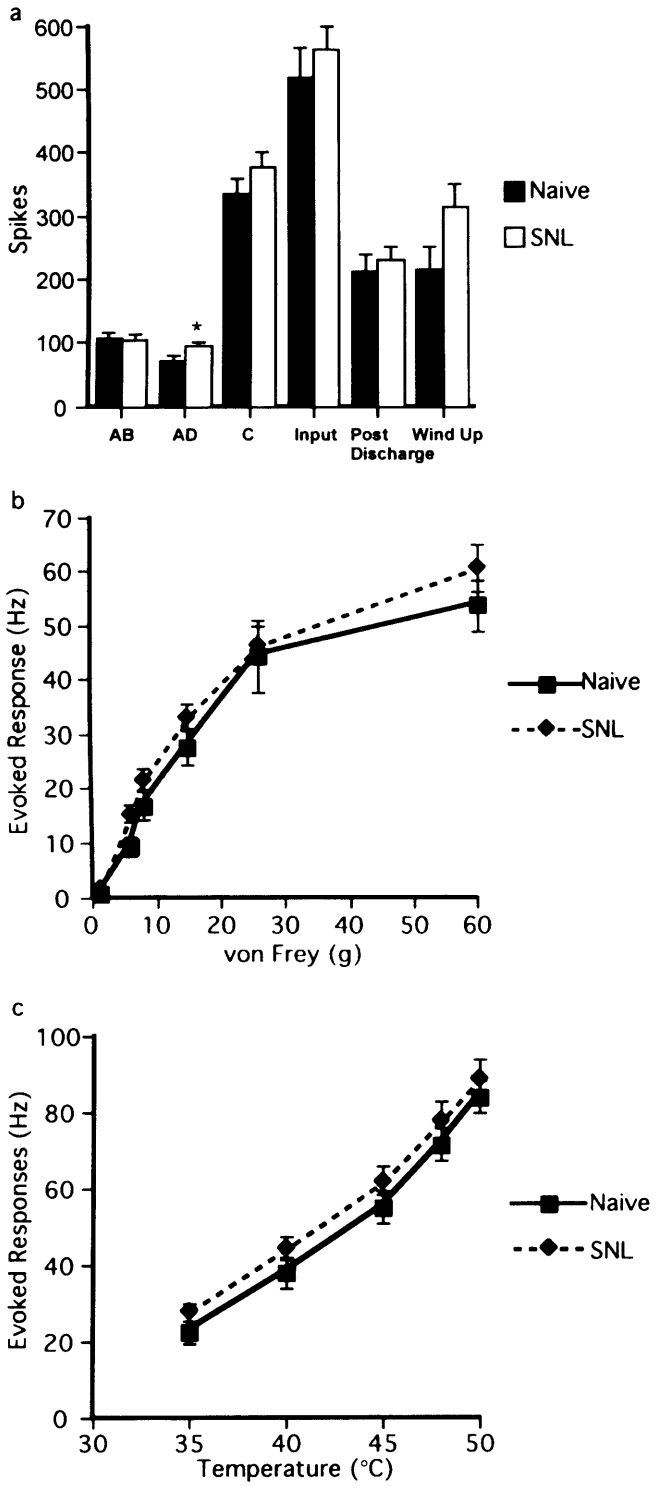
Table 3.1 *Comparison of the mean dorsal horn neurone depth recorded from, C-fibre threshold and electrically evoked Aβ-, Aδ- and C-fibre responses, input, post-discharge and wind-up between naïve rats and SNL rats. Data are expressed as mean ± SEM with * denoting statistical significance (P<0.05) between the groups.*

	NAÏVE	SNL
Depth of neurone (μm)	721 ± 121	698 ± 87
C-Fibre Threshold (mA)	1.76 ± 0.1	1.9 ± 0.2
Aβ-Fibre-Evoked Response (AP)	107 ± 10	105 ± 7
Aδ-Fibre-Evoked Response (AP)	71 ± 8	98 ± 6*
C-Fibre-Evoked Response (AP)	336 ± 24	377 ± 25
Input	518 ± 47	562 ± 36
Post-Discharge (AP)	212 ± 28	231 ± 21
Wind-Up	215 ± 36	313 ± 36

Presented in graph form in Figure 3.8

In terms of responses to natural stimuli, neurones in both groups of rats neatly coded the strength of the stimulus so the number of action potentials fired in a 10s period increased with stimulus strength (Figure 3.7 and figs 3.8b-c). Figures 3.8b-c show that the stimulus-response functions for mechanical stimuli (b) and thermal stimuli (c) were not different between the two groups of rats; neurones showed consistent responses to stimuli regardless of the presence of nerve injury and experimental pain behaviours.

Figure 3.7 *Responses of dorsal horn neurones in naïve and SNL rats to stimulation of their peripheral receptive fields. Responses evoked by repeated short pulse (2ms) electrical stimulation (a), 10s mechanical stimulation (b) and 10s thermal stimulation (c) are illustrated (responses to natural stimuli were normalised by subtracting basal firing from the total number of action potentials fired during the 10s stimulus duration). Data are expressed as mean ± SEM with * denoting statistical significance ($P<0.05$) between the groups.*



3.4 DISCUSSION

Since its development in 1992 (Kim and Chung, 1992), the SNL model of neuropathic pain has been widely used in different animals, contexts and research settings to increase the understanding of the complex peripheral and central processes underlying neuropathic pain. I have herein demonstrated successful replication of this model through the careful and tight ligation of the left L5 and L6 spinal nerves in male Sprague-Dawley rats weighing approximately 130g. Operated rats went on to quickly develop evoked behavioural hypersensitivities in their hindpaws ipsilateral to the side of nerve injury, as has previously been reported (Kim and Chung, 1992, Chapman et al., 1998b, Suzuki et al., 2000). These behavioural responses, reminiscent of hypersensitive and allodynic phenomena in neuropathic pain patients, were significantly different from contralateral PWD responses by post-operative day 2.

Afferents in the spinal nerves are comprised of primary sensory neurones, whose normal impulse generation sites lie cutaneously (as is the case for L5/6) in the sensory receptors of their distal terminals. Tightly ligating or lesioning these nerves disconnects the neurones from their receptors (where, as described, action potentials are generated), yet discharges emanate from these neurones regardless within 13 hours of injury (Liu et al., 1999, Liu et al., 2000). This points to an alternative source of impulse generation, which, given the spontaneity of the discharge, is independent of any stimulus. Within 10 minutes of nerve injury in the SNL model, ERK, an extracellular MAP kinase that plays a critical role in intracellular signal transduction and neuronal plasticity, activates within damaged neurones and sets in motion a series of neuronal changes that lay the foundations for altered sensory processing (Zhuang et al., 2005). After six hours, this enzyme switches its activation to surrounding microglia where it remains active for many days, corroborating microglial support of the pain phenotype before switching on in astrocytes after a delay of several weeks. This temporal and primitive pattern of ERK activation after nerve injury likely influences Na^+ channel redistribution and ectopia along the damaged neurone, and has a direct bearing on hypersensitive behaviours (intrathecal injection of an inhibitor at various time points after SNL significantly reduces PWD frequencies to mechanical stimuli (Zhuang et al., 2005)). In addition, behavioural hypersensitivities directly correlate with ectopic activity in the damaged nerve, at least during the early stages of

injury and pain (Sun et al., 2005). Thus the early behavioural results observed in my studies are at least partly the result of altered injury-induced intracellular signalling cascades within damaged neurones that affect ion channel plasticity and ectopic activity. Only 'partly' however since there are at least two peripheral components to neuropathic pain, one of which does not depend on hyperexcitability in damaged neurones but which depends instead on intact peripheral neurones accessing an altered central nervous system.

Ipsilateral PWD responses to all mechanical stimuli peaked after day 2, developing beyond their initial level over the course of the post-operative testing period, as reported previously (Chapman et al., 1998b). It has already been said that ectopic activities in neurones underlie behavioural sensitivities during the early phase of neuropathic pain, yet these begin to lose impetus as the days progress (Sun et al., 2005) suggesting that other factors come into play to support and exacerbate the ongoing behaviours. Indeed, initial barrages of input into the spinal cord sensitise the dorsal horn and unleash NMDA receptors so that further incoming sensory inputs are amplified (Dickenson, 1995b), whilst facilitatory influences from supraspinal structures activate after nerve injury in a time-dependent manner (Burgess, 2002). The eventual levelling off of effect by day 14 (and stabilisation for up to 4 months observed in other studies (Kim and Chung, 1992)) suggests that the injury-altered nervous system reaches a new equilibrium, hence ectopic activities in peripheral nerves settle down (Sun et al., 2005), descending and central amplification of input becomes steady and proficient (Burgess, 2002), and microglia activate at multiple foci (Zhuang et al., 2005).

Effects of the readjusted nervous system are unilateral however, since the contralateral paw had significantly lower withdrawal responses to all stimuli on each of the testing days. Other groups have reported mild hypersensitivity in the uninjured paw beyond day 14 (Takaishi et al., 1996, Tabo et al., 1999) which might reflect mirror-image pain seen in the clinic after nerve injury (Bonica, 1979), yet neither this time point, nor the evoking stimulus (vF 19g) were tested in the present study. In addition, I did not measure PWD responses to thermal stimuli as others have previosuly (Kim and Chung, 1992, Kontinen et al., 1998, Roytta et al., 1999). Although results relating to thermal hypersensitivities conflict, many conclude that

the ipsilateral paws of SNL rats do not show increased response frequencies to a radiant heat source (Kontinen et al., 1998, Roytta et al., 1999). Differences in the development of behavioural hypersensitivities may therefore depend on stimulus modality, possibly due to different transduction mechanisms being affected (if at all) to different extents by nerve injury. It has already been said that L5/6 ligations gives rise to a novel 'modified rapidly adapting mechanoreceptor' that innervates the affected paw, and so this in particular may influence responses to mechanical stimuli after nerve injury (Na et al., 1993).

In this chapter I have presented data from two separate populations of SNL rats to demonstrate reproducibility of effect. It is interesting to note that behavioural testing of other SNL rats using the same stimuli on the same post-operative days but by different testers, also yield similar patterns of behaviour and significance, although the magnitude of responses shown by the rats differ according to the experimenter (Chapman et al., 1998b, Matthews and Dickenson, 2001b, Rahman et al., 2006). The experimenter represents the biggest source of variability in behavioural results from a consistent group of animals (Chesler et al., 2002). I alone carried out behavioural testing on my operated rats, yet as the results show, small variabilities were seen both within groups, and between groups and studies. Although outward signs of behaviour (for example gait, posture and temperament) seemed very consistent between rats, some tended to guard their affected paw more than others, whilst others showed a higher tendency to weight-bear on their contralateral side at rest (N.B. this weight shift does not influence PWD reflexes (Kauppila et al., 1998)).

One of the most puzzling features of neuropathic pain in humans is the wide variability in reported pain intensity, suffering and treatment responsiveness from patient to patient even when the underlying nerve injury seems to be identical. In humans, two large factors that precipitate variability are heritability and past experience, yet even with the elimination of these factors in animal studies (our rats are bred to be genetically identical and are reared in the same environment under the same conditions with equivalent access to food and water), a range of responses to the same stimulus are seen. The SNL model was chosen for its said reproducibility; instructions for tight ligations of the whole nerve (L5/6) means that in each rat, the key (and perhaps lone) source of damage variability lies in the contribution that each

spinal segment makes to the sciatic nerve, something that can differ depending on subtle anatomical differences between rats that are beyond the boundaries of control. In this model, the hindlimb afferents are cut across far more proximally than in other peripheral nerve injury models so that ligations are made just short of the DRG. The responses of neurones to ligation, and also responses of dorsal horn neurones and spinal glia to the damage, depends on the precise location of the nerve injury with respect to its DRG, hence even slight, millimetre alterations in the position of the lesion may affect the pain phenotype and associated behaviours. Nevertheless, whilst this serves to remind us of the multiplicity of factors that can affect responses to nerve injuries, the real point is to emphasise that in spite of these, the integrity of the SNL model remains, producing enduring, replicable and statistically significant behavioural hypersensitivities in the ipsilateral paw as demonstrated.

It is both fascinating and remarkable that such a low-threshold stimulus such as vF 1g, which causes minimal withdrawal responses in the contralateral paw, can evoke brisk withdrawal, shaking and occasional licking of the injured paw after SNL surgery. Low-threshold inputs are transduced and relayed from the periphery to the spinal cord along myelinated A-fibres. The vast majority of neurones that show ectopic firing after SNL have axons of fine A-fibre calibre (Han et al., 2000), and alongside voltage-gated Na^+ channels, up-regulated HCN channels are thought to underlie this increased level of activity (Chaplan et al., 2003). In these experiments, SNL rats had significantly greater basal neuronal activity relative to control rats, as described previously (Chapman et al., 1998b, Suzuki and Dickenson, 2006). It was therefore necessary to normalise responses of dorsal horn neurones to natural (i.e. mechanical and thermal) stimuli in this group of rats by subtracting the number of action potentials spontaneously fired during each 10s recording period (typically 25-35 action potentials) from the total number of action potentials fired upon stimulus application.

Low-threshold inputs are ordinarily subject to inhibitory control in the dorsal horn of the spinal cord such that they are prevented from accessing the nociceptive system. Pathological disruptions of local spinal cord inhibitions may however enable innocuous inputs to relay in superficial laminae onto ascending pathways that mediate pain sensation (Torsney and MacDermott, 2006).

Inhibitory GABAergic and glycinergic interneurones constitute between 30-40% of the neuronal population in this region of the spinal cord (Todd and Sullivan, 1990), yet after nerve injury, inhibition is imperilled by reductions in GABA and its synthesising enzyme GAD65 (Ibuki et al., 1997, Moore et al., 2002). Furthermore, blocking local spinal cord inhibition mimics behavioural hypersensitivities seen with chronic pain (Yaksh, 1989) and facilitates low-threshold input to the dorsal horn (Baba et al., 2003). Whole-cell recordings suggest the existence of polysynaptic A-fibre inputs onto NK1-receptor expressing lamina I cells that are usually under strong inhibitory control (Torsney and MacDermott, 2006). This pathway, which is predominantly Aβ-fibre mediated and dependent on NMDA receptors for activation, is capable of inducing behavioural hypersensitivities and is revealed after nerve injury, possibly as the result of a functional loss of inhibitory interneurones (Sugimoto et al., 1990, Dickenson, 1997, Moore et al., 2002) and shifts in anion gradients that render remaining 'inhibitory' interneurones as excitatory (Coull et al., 2003). In addition, nociceptive C-fibres that usually have activation thresholds higher than 1g (Leem et al., 1993a) may become peripherally sensitised to the extent that they too start conveying low-threshold inputs to the spinal cord. Together, the A- and C-fibre induced transformation of central NS neurones into WDR neurones explains why rats respond to low-threshold inputs and cooling stimuli in an exaggerated way after SNL surgery.

In line with the known alterations in A-fibre responsiveness and nervous system access, my electrophysiological characterisations of dorsal horn neurones showed a statistically significant greater Aδ-fibre response to peripheral electrical stimulation in SNL animals compared with naive animals. This however was the only translational consistency between the behaviour and electrophysiology studies within a group, since in the latter, all other electrical-, mechanical- and thermal-evoked responses were comparable between the two groups of rats. Thus stimulus-response functions overlay one another, with no gain, loss or alteration in coding ability between the populations. In a similar way, it has previously been shown that L4-L6 dorsal root constriction injuries do not alter dorsal horn neuronal responses after 5 weeks even though behavioural hypersensitivities are maximal at this time (Tabo et al., 1999), whilst in the PSL model, changes in spinal neuronal responses have

temporal profiles out of sync with behavioural hypersensitivities (Takaishi et al., 1996). These apparent idiosyncracies can be explained. First of all, behavioural and electrophysiological tests measure threshold versus suprathreshold responses. Secondly, behavioural responses, unlike those given in electrophysiology, represent cumulative whole-system responses that are the product of integrated internal and external influences. Although WDR neurones (the cells recorded from electrophysiologically) comprise over half of the neurones of the spinothalamic tract, a major pain signalling pathway (Chaouch et al., 1983), there are also contributions from NS neurones, non-nociceptive neurones, as well as other tract and structural influences. Furthermore, the nociceptive withdrawal reflex inevitably depends on motor responses that are triggered in the ventral horn, which is itself under vast modulation from many sources (monoaminergic neurones from the brainstem for example exert considerable control over both the sensory and motor systems).

Whilst it is impossible to directly marry results from behavioural and electrophysiological studies, correct interpretation of apparent 'discrepancies' (i.e. dorsal horn responses to low-threshold input did not show increased firing as might be surmised from the behavioural data) may actually draw attention to important pain mechanisms. Taking this example as a starting point, observations that low-threshold stimuli did not trigger hyper-responses electrophysiologically (contrary to expectation, a lower proportion of neurones in the SNL rats responded to the lowest stimulus used compared to naïve animals, which is similar to reports from other nerve injury models (Laird and Bennett, 1993)) indicates that some form of compensation must occur to increase the eventual behavioural response of the animal to this stimulus. Damage to the PNS results in a substantial loss of primary afferent neurones, which means that stimulus-evoked inputs are conveyed along fewer fibres (Castro-Lopes et al., 1990). In the SNL model, the injuries sustained almost completely abolish input into the corresponding spinal segments, as well as reducing inputs into adjacent segments (it has been estimated that after SNL, there is a 35% deficit in afferent input to the ipsilateral L4 segment (Besse et al., 1991).

My own personal observations made during electrophysiological recordings from SNL animals confirm the relative scarcity of responsive neurones in the L5/6 segments, with a greater success of isolating neurones in spinal cord sections rostral

to the affected segments. Thus, if dorsal horn neurones are receiving much less input from the targeted area (i.e. the hindpaw) yet respond 'normally' to stimuli, then information from the remaining afferents must be amplified at some level. If we once again refer to the fact that peripheral ectopic drive declines in the face of maintained behavioural hypersensitivities, then the concept of central magnification is emphasised. Electrophysiologically this model is responsive to the inhibitory effects of carbamazepine, NMDA- and $5HT_3$- receptor antagonists, which indicates that compensatory facilitatory mechanisms may operate at peripheral, spinal and supraspinal levels respectively (Chapman et al., 1998a, Suzuki et al., 2002b). These compensatory mechanisms likely explain how positive and negative symptoms, representing enhanced sensations and sensory deficits, often co-exist in nerve-injured patients (N.B. incidentally, in searching for recordable cells, I noted that many dorsal horn neurones were encountered in the ipsilateral spinal cord that did not have peripheral receptive fields, yet fired spontaneously and in an irregular way, which is further indication of this paradoxical pairing).

In conclusion, the behavioural results that I have obtained which show a rapid onset of mechanical and cooling hypersensitivities in the ipsilateral paw that develop and are maintained throughout the post-operative testing period, fit with the original behavioural results (Kim and Chung, 1992) and others obtained since (Chapman et al., 1998b, Suzuki et al., 2000), which indicates successful replication of the injury and experimental pain phenotype. Electrophysiological recordings from neurones whose response profiles, depth within the spinal cord and wind-up capacity were suggestive of WDR neurones, highlighted a difference in Aδ-fibre responses to electrical stimulation between SNL and naïve rats, with all other responses to peripheral stimuli being comparable. The electrophysiological and behavioural data can be reconciled with explanations of central compensation and amplification that are known to enhance responses after nerve injury. My validation of this nerve injury model means that it can be applied to other studies to look confidently at the contribution of brainstem and descending influences to neuropathic pain.

4. THE BRAINSTEM AND NEUROPATHIC PAIN: EXPERIMENTS WITH LIGNOCAINE (expanded from Bee & Dickenson, 2006)

4.1 ENDOGENOUS MODULATION OF PAIN

4.1.1 EARLY V. PRESENT THEORIES RELATING TO PAIN PROCESSING AND MODULATION

In the 17th century, the scientist and philosopher Rene Descartes postulated several theories on the nature of pain, and in one particular treatise, *De l'homme*, he wrote about 'delicate threads' that conduct peripheral pain signals to an alert and responsive brain, much like the 'pulling of one end of a rope' that results in a bell sounding (Descartes, 1644). The famous illustration of a man with his foot in a flame that accompanies this theory is labelled A through to F, with A representing the sensation of pain in the foot, and F denoting the centre in the brain that the pain signal arrives at for interpretation (see Figure 4.1). This Cartesian view of pain was farsighted in at least one respect, since we now know the 'delicate threads' to be a distinct and separable class of peripheral nociceptive fibres that are distributed throughout the body (Sherrington, 1906); detection of a noxious stimulus begins with nociceptors expressed by these fibres, which, upon activation, transduce the stimulus energy into electrical signals. An afferent volley discharges these signals to the spinal cord to elicit post-synaptic responses and eventual perception in the brain. This point may also accede Descartes' reasoning, yet in his linear model, pain is viewed as a submodality of touch sensation that is promoted to a concept by a decoding brain that is impervious to affect, environment and contextual influences. The 1:1 relationship between stimulus and response that was originally thought to govern the hard-wired route from periphery to brain is now known to be an extreme over-simplification of what actually happens.

The idea of pain as a fixed system eventually gave way to a more modern way of thinking, and by 1965, Melzack and Wall had proposed a plastic and integrative model of pain. Their Gate Control Theory suggested that the spinal cord acts as a gate-keeper to incoming pain signals, differentially enhancing or suppressing

synapses between first order nociceptive neurones and second order 'pain transmitting' neurones befitting of the circumstance (Melzack and Wall, 1965). The theory additionally expanded on earlier findings by Sherrington that higher centres of the brain could influence the transmission of nociceptive information (Sherrington, 1906), an idea empirically confirmed in 1954 by Hagbarth and Kerr, who proposed a descending system that could modulate pain (Hagbarth and Kerr, 1954). Thus the Gate Control Theory took the focus away from the source of pain and shifted it instead to the CNS, insinuating meanwhile that the brain had final control over the volume of pain. As such this theory embraced the physiological, psychological and socio-cultural factors that are now known to influence pain and are inherent to its current definition (See Section 1.1). Moreover, this new way of thinking had a profound sway on the management of pain and opened up a multi-faceted approach to its treatment, pharmacological and otherwise.

The advent of neuroimaging and analytical techniques pushed the frontiers of pain research further still through the identification of a 'pain matrix' in the brain that is made up of areas involved in human nociceptive processing (for a review see (Brooks and Tracey, 2005)). This matrix is fluid and takes on different patterns of activity from one person and pain state to the next, yet there is a pharmacologically sensitive core made up of the thalamus, anterior cingulate cortex, insula and somatosensory cortices (S1 and S2) where activity is taken to indicate sufficient nociceptive processing for pain perception (Wise et al., 2002, Rogers et al., 2004). In addition, midbrain and brainstem clusters show increased activity during pain and deactivate in response to known analgesics (Iannetti et al., 2005). Together with diffusion tractography techniques that highlight anatomical connections between cortical and brainstem regions, these studies in humans illustrate a physical route by which contextual relevance, cognitive set and mood converge to influence pain and the spinal processing of sensory information in a top-down fashion (Hadjipavlou et al., 2006).

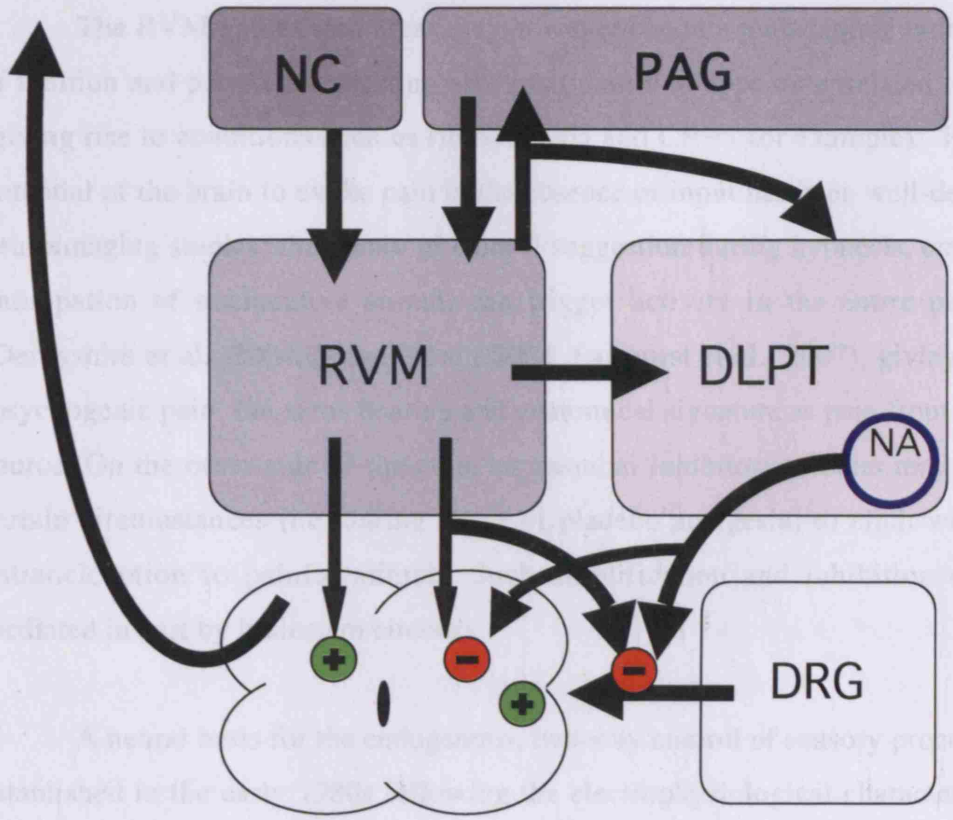
Figure 4.1 *Descartes' illustration of pain processing, taken from De l'homme (Descartes, 1644)*

4.1.2 PAIN AND THE BRAIN

The PAG and RVM, which lie in the midbrain and brainstem respectively, are key structures in the descending modulatory repertoire. Early indications that these areas could influence nociception and pain came with the finding that electrically stimulating the PAG of rats caused no overt behavioural signs of distress during an otherwise 'painful' procedure (Reynolds, 1969). Thereafter it was shown that electrically stimulating the PAG could block intractable pain in human subjects (Hosobuchi et al., 1977), and that stimulation-produced analgesia (SPA) could also be triggered downstream of the PAG in the RVM (Fields and Basbaum, 1978). The PAG receives information from the prefrontal cortex, the insular cortex, the amygdala and hypothalamus, and is reciprocally connected with the RVM. The PAG collects, processes and integrates ascending nociceptive inputs with descending information from the diencephalon and limbic forebrain, and then projects via the RVM to the dorsal horn. In turn, the RVM, a crucial junction and relay site in this descending control, receives information from autonomic, homeostatic, affective and sensory systems and projects directly to the spinal cord via the dorsolateral funiculus (DLF) and indirectly via the dorsolateral pontine nucleus. A noradrenergic circuit arising from the dorsolateral pontine tegmentum (DLPT) in the brainstem influences the

output of the RVM, as well as modulating spinal cord processing of its own accord (Yaksh, 1979, Proudfit, 1988). Together, descending projections from the RVM and the DLPT alter the tone of sensory signals in the spinal cord via direct presynaptic actions on the dorsal horn terminals of primary afferent fibres, postsynaptic actions on second order projection neurones, and also via actions on intrinsic interneurones within the spinal cord (Fields et al., 1991).

Figure 4.2 *Schematic diagram showing the anatomical links between the nucleus cuneiformis (NC), PAG, RVM, dorsolateral pontine tegmentum (DLPT) and dorsal horn of the spinal cord.*



The emphasis of early experimental work relating to the descending system was on its inhibitory capabilities, yet gradually the hub of research shifted to encompass descending *facilitatory* controls when it became apparent that the brain could bi-directionally modulate spinal cord activity; the net output of the descending facilitatory and inhibitory pathways from the RVM determines whether neuronal

activity in the spinal cord is enhanced or suppressed. Under normal conditions, a finely set equilibrium likely exists between the opposing controls to support a level of spinal gain that is large enough to detect noxious stimuli, yet not large enough to permit excessive sensory transmission. However, the balance is variable and may weight in favour of either facilitation or inhibition. Ergo, whilst the RVM may serve a protective, or 'masking' role during some pain states by increasing its inhibitory output, for example during inflammatory pain and potentially during the early stages of cancer, it may alternatively increase its facilitatory drive so that sensory transmission and eventual perceptions of pain are enhanced and sustained. In this particular circumstance, which may occur during neuropathic pain, ongoing feedback onto brainstem structures primes the system and amplifies spinal nociception so that perceived pain is greater (Suzuki et al., 2002b).

The RVM and related areas may however become maladaptive independently of ignition and permit long-lasting abnormal pain that appears unrelated to a source (giving rise to conditions such as fibromyalgia and CRPS for example). Indeed, the potential of the brain to evoke pain in the absence of input has been well-described in neuroimaging studies; the power of painful suggestion during hypnosis, empathy and anticipation of nociceptive stimuli can trigger activity in the entire pain matrix (Derbyshire et al., 2004, Singer et al., 2004, Fairhurst et al., 2007), giving so-called 'psychogenic pain' the same bearing and anatomical signature as pain from a physical source. On the other side of the coin, supraspinal inhibitory circuits may prevail in certain circumstances (i.e. during stress or placebo analgesia) to elicit wide spread antinociception to painful stimuli. Such amplification and inhibition of pain is mediated in part by brainstem circuitry.

A neural basis for the endogenous, two-way control of sensory processing was established in the early 1980s following the electrophysiological characterisation of three classes of cells in the brainstem that showed different firing patterns related to their responses to noxious heat (Fields et al., 1983a, Fields and Heinricher, 1985). Thus, as described in Section 1.3.3.3, On cells showed a burst of activity related to noxious stimulation of the tail, Off cells showed a pause in activity prior to the same stimulus and withdrawal response, whilst neutral cells showed no identifiable change in activity that could be correlated with the nocifensive withdrawal reflex. A large

body of evidence has since suggested that Off cells are the RVM's inhibitory output, whilst On cells are the pronociceptive output neurones (Heinricher et al., 1994, Neubert et al., 2004).

4.1.3 PHARMACOLOGICAL MANIPULATION OF THE RVM

The RVM can be pharmacologically manipulated by various agents so that its output is dominated by either facilitatory or inhibitory neurones (Heinricher et al., 1991, Heinricher et al., 1992, Heinricher et al., 2001a), with some agents capable of producing a dose-related bidirectional effect when applied within the RVM. For example, at low doses, glutamate facilitates spinal nociception, whereas at higher doses it inhibits dorsal horn neuronal responses (Zhuo et al., 2002). Likewise, extremely low doses of neurotensin acting at medullary NT1 receptors activate On cells and produce pronociceptive behaviours, whereas higher doses additionally recruit Off cells and produce behavioural anti-nociception (Neubert et al., 2004). Low doses of these agents promote pain, which suggests that descending facilitations may only switch to inhibitions in extreme situations. Nevertheless, the differing susceptibilities of the On and Off cells to these various agents can be used as a tool to investigate the mechanisms by which the brainstem communicates with the spinal cord in both normal and pathophysiological states.

The local anaesthetic lignocaine, which inhibits the generation and conduction of action potentials by non-selectively blocking voltage-gated Na^+ channels, has been widely used to study the role of discrete brain regions in descending modulation. Its microinjection into the RVM has been shown to prevent secondary hyperalgesias that are otherwise induced by mustard oil application to the upper leg in rats (Kincaid et al., 2005), and was also able to fully reverse the behavioural hypersensitivities established by SNL surgery (Pertovaara et al., 1996). The MOR expressing cells, which are thought to represent the population of facilitatory neurones in the RVM (more later) are the likely substrate of these behaviours since in rats, their specific ablation reverses the behavioural manifestations of pain that are initiated by nerve lesions (albeit in a time-dependent manner) (Buhler et al., 2004). Furthermore, behaviourally correlative recordings made from the RVM in Kincaid's study showed that only On cells increased their activity during mustard oil application. A plausible

explanation for the effects seen in both these and similar studies, (Ren et al., 1990) (Pertovaara et al., 1996, Chen et al., 2004, Taylor et al., 2007) is that circumscribed inactivation of the RVM interrupts a predominant facilitatory drive. However the impact of this system on spinal neuronal activity has not yet been studied. It is therefore my intention to provide a neuronal correlate to this behavioural data, to investigate whether, and if so how, pharmacologically preventing action potential propagation in some RVM neurones (and fibres of passage therein) with the local anaesthetic lignocaine impacts spinal neuronal processing to a range of innocuous and noxious mechanical and thermal stimuli, as well as supra-threshold electrically-evoked responses, in both normal anaesthetised rats, and rats that had received SNL surgery and displayed appropriate behaviours two weeks previously.

4.2 METHODS

4.2.1 SNL SURGERY, BEHAVIOURAL TESTING AND *IN VIVO* ELECTROPHYSIOLOGY

25 naïve animals, and 26 animals that had received nerve injuries 14-17 days previously were used in the electrophysiology study. SNL surgery, subsequent behavioural testing and *in vivo* recordings were carried out as described in Sections 2.3, 2.4, 2.5 and 3.2. However, instead of only characterising dorsal horn neuronal responses to peripheral stimuli, in the present study neurones were monitored both before and after injection of lignocaine into the RVM. Thus, following stabilisation of a neurone's electrophysiological responses, which was usually confirmed by three control studies (with less than 10% variation between studies for all measures taken as acceptable), lignocaine was injected into the RVM. The testing cycle resumed, and responses to all stimuli were once again recorded at 20-minute intervals over a 120-minute period.

4.2.2 DRUG ADMINISTRATION

With a level skull and following stereotaxic co-ordinates adapted from the rat brain atlas of Paxinos & Watson (2005), a Hamilton syringe loaded with 2% lignocaine (=20μg/ml) was unilaterally driven through a finely drilled hole in the skull into the RVM and 0.8μl was injected into the brain tissue (=16ng lignocaine). The syringe remained clamped in its vertical position for the duration of the experiment (both before neurone isolation and after injection) so as to not disturb the isolated neurone and to minimise tracking of the drug back up the needle, which might have otherwise occurred upon withdrawal of the syringe.

4.2.3 HISTOLOGY

As described in Section 2.7 the location of the end of the injection needle was plotted onto corresponding representative sections that included the RVM (centred around bregma –9.84). Data from injection sites outside the RVM had little effect on spinal responses, and while serving as positive controls in terms of site specificity,

were excluded from the overall analysis. Meaningful correlations between locations of injection site and eventual effect (i.e. inhibition or facilitation of spinal responses) are not evident.

4.2.4 DATA ANALYSIS

Behavioural data are presented as mean±SEM paw withdrawal response to von Frey 6g and acetone on each post-operative day tested (ipsilateral paw versus contralateral paw). Statistical significance was calculated using non-parametric Wilcoxon matched pairs tests.

Electrophysiological raw data are presented as mean±SEM response (number of spikes evoked by a given stimulus) for averaged pre-drug controls, and for responses at 20-minute intervals after the injection of lignocaine into the RVM (up to and including the 120-minute time point). Neurones were divided into two groups based on the time-related direction of changes in the majority of their evoked responses after injection of lignocaine into the RVM. These changes had to consistently exceed 15% of the control. Statistical analyses were performed on raw data using two-way analysis of variance (ANOVA) for responses to mechanical and thermal stimuli, and if significant, Bonferroni post-hoc tests were performed. For responses to electrical stimulation, a one-way analysis of variance was used followed by Dunnett's post-hoc multiple comparisons test for significant values.

4.3 RESULTS

4.3.1 EXPLANATION OF ELECTRICAL MEASUREMENTS

Before detailing the results, a small note on what the different electrical measurements represent may be useful for their interpretation:

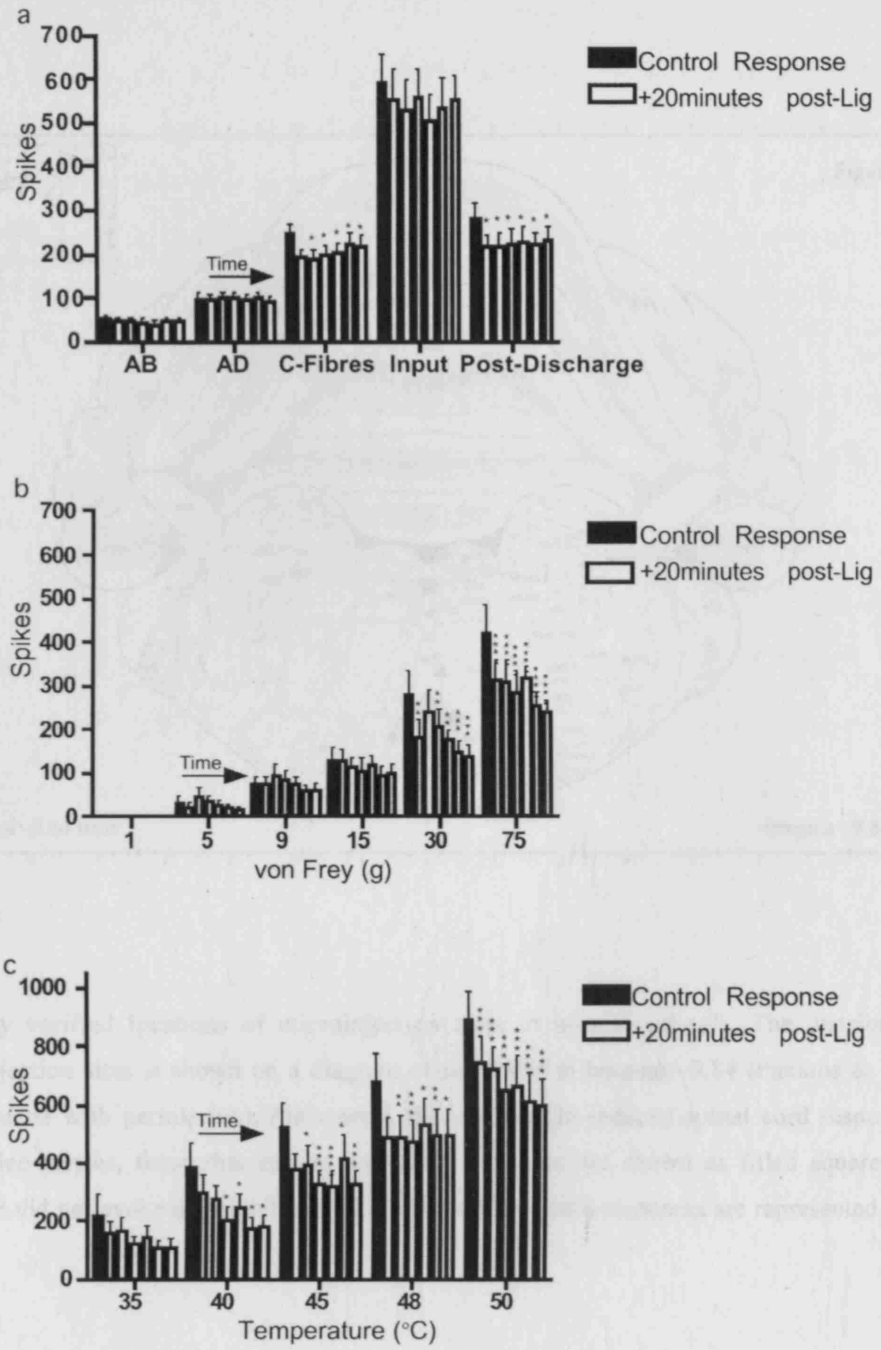
Input represents the post-synaptic C-fibre-evoked dorsal horn neuronal response following the first of the 16 electrical stimuli in the electrical train. It gives a measure of resting pre-synaptic activity (taken as total neuronal responsiveness upstream of the neurone under study, therefore including afferent excitability, activity at terminals and interneurones) and transmitter release in the absence of potentiation. The baseline 'input' response given numerically and graphically in this and other studies, was calculated as the number of C-fibre-evoked action potentials produced by the first stimulus (i.e. initial baseline response) multiplied by the total number of stimuli (16). **Aβ-**, **Aδ-**, and **C-fibre responses** denote the number of action potentials respectively generated in the spinal cord as a consequence of Aβ-, Aδ-, and C-fibre activity following electrical stimulation of their peripheral receptive field. The Spike 2 Data Capturing software separates out which incoming action potentials are carried by which fibre according to their latency to arrive in the spinal cord following generation (given the different conduction velocities of the fibres). Thus, those arriving within 20ms of peripheral stimulation are attributed to Aβ-fibres, Aδ-fibres = 20-90ms, and C-fibre-evoked responses were taken as those recorded 90-300ms after electrical stimulation. Neuronal responses that trail behind between 300-800ms post-stimulus are quantified as **post-discharge**, and represent spinal cord hyperexcitability that results as a consequence of repetitive stimulation of the neurone. Activity-dependent hyperexcitability can additionally be measured as **wind-up**. This value is calculated as the difference between the total number of action potentials at C-fibre latency produced by the train of 16 electrical stimuli, and 'input' as defined and calculated above. Therefore, if the first electrical stimulus elicited 15 C-fibre-evoked action potentials, and the *total number* of C-fibre-evoked action potentials recorded after 16 stimuli was 350, then wind up = 350 – (15x10) = 200. If each post-synaptic response was independent of previous activity, then in the example above, the cumulative number of action potentials evoked by C-fibres after the 16 electrical stimuli should

theoretically be 150. However, temporal summation of action potentials, and post-synaptic hyperexcitability amplify responses, so wind-up is a measure of the additional action potentials recorded above the predicted baseline level.

4.3.2 THE EFFECTS OF INTRA-RVM LIGNOCAINE IN NORMAL ANIMALS

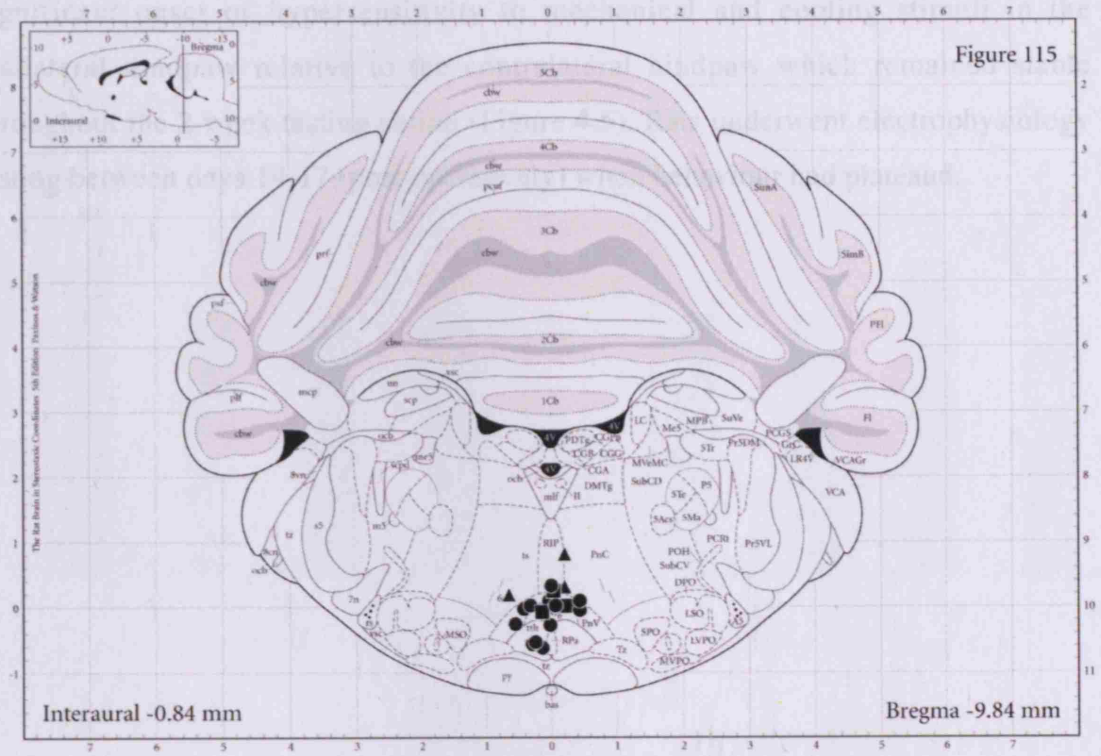
In the 25 normal rats studied, 64% (n=16) of neurones showed reduced responses to many of the applied stimuli after local anaesthetic injection in the RVM (figs 4.3a-c). C-fibre responses to electrical stimulation of the peripheral receptive field became significantly inhibited at t=40 and remained reduced (relative to pre-drug control responses) until the end of the experiment (Figure 4.3a). A-fibre responses to peripheral electrical stimulation did not significantly alter following lignocaine injection into the RVM and neither did input or post-discharge. With respect to mechanical punctate stimuli, responses to suprathreshold noxious stimuli (vF 30g & 75g) became significantly reduced after lignocaine's injection into the RVM, yet responses to low threshold stimuli did not significantly alter in this population in line with the lack of effect on A-fibre responses (Figure 4.3b). The time course of inhibitions in response to these high threshold stimuli were similar, manifesting 20 minutes after lignocaine injection and remaining reduced until the end of the 120 minute testing period. A similar inhibitory trend was seen with responses to thermal stimuli; responses to lower temperatures were either not significantly inhibited, or less significantly inhibited ($p<0.05$) than responses to higher temperatures ($p<0.01$ and 0.001). The onset of inhibition was more immediate for responses to 45°C, 48°C and 50°C relative to responses to 40°C (Figure 4.3c). Of the 9 neurones whose responses did not decrease following the injection of lignocaine into the RVM, 6 had significantly increased responses to many of the applied stimuli post-injection, whilst responses of the remaining 3 neurones were not affected by the injection of lignocaine into the RVM. The histologically verified injection sites corresponding to these neuronal responses are plotted in Figure 4.4.

Figure 4.3 *The effects of intra-RVM lignocaine on the response characteristics of dorsal horn neurones in 16 out of 25 of naïve animals tested.*



Intra-RVM lignocaine decreased dorsal horn neuronal responses to peripheral electrical stimulation, in particular C-fibre activity and post-discharge *(a)* as well as responses to peripheral noxious mechanical stimulation *(b)* and thermal stimulation *(c)* in a time-dependent manner in 16 of the 25 naïve animals. Black solid bars represent mean pre-drug control responses, while each open bar thereafter represents the mean response recorded at a 20 minute interval after the injection of lignocaine into the RVM, up to and including the 120 minute time point (*P<0.05, **P<0.01, ***P<0.001).

Figure 4.4 *Location of injection sites within the brainstems of normal animals.*

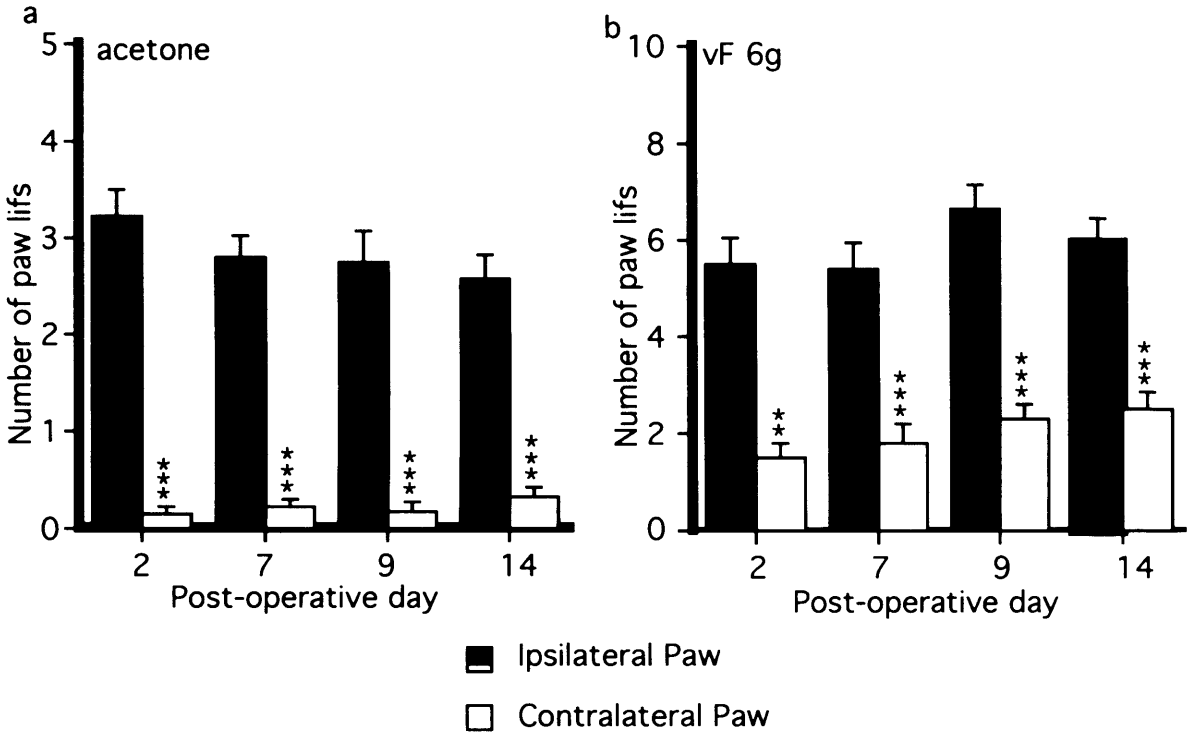


Histologically verified locations of microinjection sites in normal animals. The distribution of lignocaine injection sites is shown on a diagram of the RVM at bregma –9.84 (Paxinos & Watson, 2005, reproduced with permission). Placements that resulted in reduced spinal cord responses are shown as filled circles, those that caused increased responses are shown as filled squares, while injections that did not evoke any significant alteration in spinal cord responses are represented as filled triangles.

4.3.3 THE DEVELOPMENT OF BEHAVIOURAL HYPERSENSITIVITIES FOLLOWING SNL SURGERY

As in Section 3.2, behavioural testing of operated animals revealed a rapid and significant onset of hypersensitivity to mechanical and cooling stimuli in the ipsilateral hindpaw relative to the contralateral hindpaw which remained stable throughout the 2 week testing period (Figure 4.5). Rats underwent electrophysiology testing between days 14-17 (post-operatively) when behaviour had plateaud.

Figure 4.5 *Induction of cooling a) and mechanical hypersensitities) following SNL surgery (n=26). Filled bars show ipsilateral PWD responses, open bars represent contralateral PWD responses.*

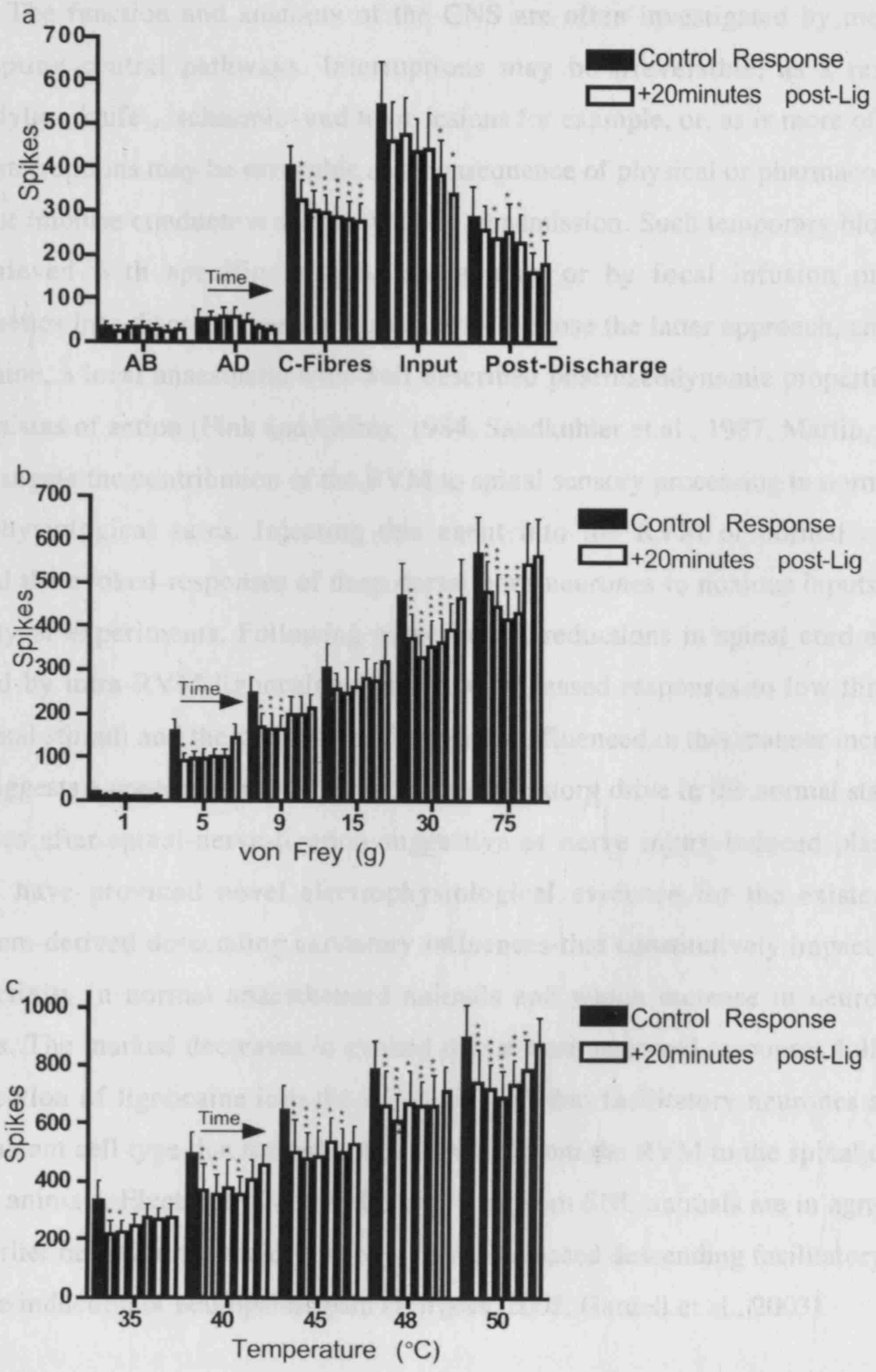


Behavioural hypersensitivity to acetone *(a)* and vF6g *(b)* as well as vF 1g, 9g and 15g (data not shown) developed rapidly in rats following SNL (n =26). Hindpaw withdrawal responses increased significantly in the paw ipsilateral to the side of nerve injury (filled bar) relative to the contralateral paw (open bar) at post-operative days 2, 7, 9 and 14 (**P<0.01, ***P<0.001). (N.B. acetone was applied to the paw 5 times, thus responses in *(a)* represent paw withdrawal responses out of a total number of 5, whilst each vF filament was applied 10 times to each paw, thus responses in *(b)* represent paw withdrawal responses out of a total number of 10).

4.3.4 THE EFFECT OF INRA-RVM LIGNOCAINE IN SNL RATS

Intra-RVM lignocaine reduced dorsal horn neuronal responses to peripheral stimuli in 21 of the 26 rats (=81%) that had previously undergone SNL surgery and accordingly displayed neuropathic behaviours. Of the responses to electrical stimulation, significant reductions in dorsal horn responses post-drug injection were seen with C-fibre evoked responses, input and post-discharge (Figure 4.6a). In contrast to normal animals, significant reductions in neuronal responses were seen in SNL animals across the whole range of natural stimuli used (Figures 4.6b-c); at each time point after the injection of lignocaine into the RVM, responses to brush were significantly reduced, and there were similarly significant reductions in response to vF 5g, 9g, 30g and 75g, as well as in response to the range of temperatures used (35°C - 50°C) over the 120-minute testing period. As in normal rats, there were isolated cases in which lignocaine induced no change in responses at all or where it caused an increase in the responses of dorsal horn neurones following its injection into the brainstem (n=3 and 2 respectively).

Figure 4.6 *The effects of intra-RVM lignocaine on the response characteristics of dorsal horn neurones in 21 out of 26 SNL animals tested.*



Intra-RVM lignocaine decreased dorsal horn neuronal responses to peripheral electrical stimulation *(a)*, noxious and non-noxious mechanical and thermal stimulation *(b)(c)* in 21 of the 26 SNL animals tested. Black solid bars represent mean pre-drug control responses, while each open bar thereafter represents the mean response recorded at a 20 minute interval after the injection of lignocaine into the RVM as denoted by the arrow (*P<0.05, **P<0.01, ***P<0.001).

4.4 DISCUSSION

The function and anatomy of the CNS are often investigated by means of interrupting central pathways. Interruptions may be irreversible, as a result of electrolytic-, knife-, ischaemic- and toxic lesions for example, or, as is more often the case, interruptions may be reversible as a consequence of physical or pharmacological block of impulse conduction and/or synaptic transmission. Such temporary block can be achieved with specific receptor antagonists or by focal infusion of local anaesthetics into discrete neuronal populations. I chose the latter approach, and used lignocaine, a local anaesthetic with well-described pharmacodynamic properties and mechanisms of action (Fink and Cairns, 1984, Sandkuhler et al., 1987, Martin, 1991), to investigate the contribution of the RVM to spinal sensory processing in normal and pathophysiological sates. Injecting this agent into the RVM of normal animals reduced the evoked-responses of deep dorsal horn neurones to noxious inputs in the majority of experiments. Following nerve injury, reductions in spinal cord activity induced by intra-RVM lignocaine further encompassed responses to low threshold peripheral stimuli and the proportion of neurones influenced in this manner increased. This suggests a predominant RVM-mediated facilitatory drive in the normal state that increases after spinal-nerve-ligation suggestive of nerve injury-induced plasticity. Thus I have provided novel electrophysiological evidence for the existence of brainstem-derived descending excitatory influences that constitutively impact spinal cord activity in normal anaesthetised animals and which increase in neuropathic animals. The marked decreases in evoked dorsal horn neuronal responses following the injection of lignocaine into the RVM suggest that facilitatory neurones are the predominant cell type that tonically project down from the RVM to the spinal cord in normal animals. Electrophysiological recordings from SNL animals are in agreement with earlier behavioural studies that suggest an increased descending facilitatory drive after the induction of neuropathic pain (Burgess, 2002, Gardell et al., 2003).

In normal animals, intra-RVM lignocaine reduced C-fibre responses to electrical stimulation (Figure 3a). In addition, there were reductions in responses to natural stimuli, although some selectivity was seen since intra-RVM lignocaine predominantly reduced responses to noxious stimuli (figs 4.3b-c). Descending facilitatory neurones may exert a large pre-synaptic influence in the spinal cord

promoting the release of neurotransmitter from nociceptive primary afferent fibres into the dorsal horn. If it were alternatively the case that these projections had a large *post-synaptic* effect, then responses to the whole range of natural stimuli, as well as post-discharge, should have decreased following lignocaine's injection into the RVM. In the corresponding studies with neuropathic animals, lignocaine did reduce neuronal responses to non-noxious stimuli. Bearing in mind these animals displayed significant hypersensitive behaviours to threshold stimuli post-operatively (figure 4.4), alterations in peripheral afferent properties may yield responses to low threshold input more amenable to descending facilitatory influences following nerve injury. Notably, in both groups of animals, intra-RVM lignocaine did not affect wind-up responses. Wind-up describes the progressive, frequency-dependent increase in the excitability of spinal cord neurones that is evoked by repetitive stimulation of C-fibres (Mendell and Wall, 1965). It is an intrinsic spinal event that is mediated via post-synaptic NMDA receptors as a prelude to central sensitisation (Dickenson, 1990), hence it can be recorded in slice preparations *in vitro* (Thompson et al., 1994, Lopez-Garcia and Laird, 1998). The inability of RVM lignocaine to modulate wind-up in my studies therefore reflects and upholds the brainstem's lack of effect on this spinal measure.

The plasma half-life for lignocaine in rats is 30 minutes (Keenaghan and Boyes, 1972). Previous studies have concluded that lignocaine attains its maximal effect within 10-15 minutes of injection, and wanes thereafter at around 45-50 minutes (Albert and Richmond, 1976, Sandkuhler and Gebhart, 1984). Another group showed that lignocaine injection into the hypothalamus did not yield significant analgesia (in the formalin test) until t=30 (Tasker et al., 1987). In the present study, the onset of lignocaine's effects (i.e. where a significant spinal change was observed following its injection into the RVM) was typically 40 minutes in the normal animals and 20 minutes in the SNL animals. The difference between onset time from one population to the next may be a consequence of lignocaine's use-dependent and frequency-dependent properties; active neurones are more susceptible to the blocking potential of local anaesthetics than resting neurones. Thus, the greater immediacy of reduced spinal cord responses seen in the SNL animals may reflect increased excitability of descending neurones targeted by intra-RVM lignocaine. Lignocaine's use-dependent properties additionally highlight the prominence of the facilitatory drive; On cells fire in a bursting manner in response to a noxious stimulus so they will

be readily silenced by lignocaine. Off cells on the other hand usually discharge at a low and ongoing rate, so these too will be silenced by lignocaine. Thus On cells lose their bursting potential, which means that the facilitatory drive is reduced, whilst Off cells are turned off and lose their inhibitory drive. The fact that the overall response (in the majority of animals) is a reduction in evoked responses in the dorsal horn of the spinal cord, means that blocking the On cells has an over-riding effect.

Whereas some behavioural studies report anti-allodynic effects in SNL animals that lasted up to 30 minutes (Pertovaara et al., 1996), others suggest that recovery from lignocaine block can take up to 1.5 hours, with partial effects lasting in excess of 2 hours (Sandkuhler et al., 1987). Other groups have reported the prolonged (i.e. >7 days) consequences of intravenous or systemic lignocaine in nerve-injured animals (Chaplan et al., 1995, Araujo et al., 2003), with abounding clinical reports and anecdotal notes suggesting a long-lasting effect of lignocaine's analgesic actions in neuropathic patients (bach et al., 1990a, Backonja and Gombar, 1992). More recently it has been shown that intra-RVM lignocaine has effects on behavioural responses in various animal models of neuropathic pain that extend up to 2 hours, peaking between 15 and 50 minutes post-injection (Taylor et al., 2007), which is very similar to my results. Given that these 'prolonged' observations are with respect to nerve-injured animals and humans, it seems that, as suggested above, the inhibitory capacity and duration of lignocaine are functions of nervous system pathophysiologies.

As well as the assumed qualitative increase in lignocaine's action in SNL animals, there may also be a quantitative element, with the actual number of descending neurones becoming blocked being greater in this population. The lag to significant effect seen for responses to some of the stimuli in the normal population may be due to the time taken for lignocaine to progressively disengage enough active neurones. Autoradiographic techniques estimate that 1μl of lignocaine spreads within a 1.7mm radius (Martin, 1991). Since the critical determinant for spread of an injected substance is its volume (and to a lesser extent the rate of infusion), the lower volume used in my study should remain within this boundary.

The inhibitory effect of lignocaine is incremental in its onset and exerts more powerful effects when injected in the RVM of SNL rats; in addition to the increased incidence of reduced neuronal responses in SNL animals relative to uninjured animals following the injection of lignocaine into the RVM, the magnitude of lignocaine's effect was also greater in these animals for responses to some, but not all, stimuli (possibly due to lignocaine's frequency- and use-dependency). In Figure 4.5a it can be seen that the responses of nociceptive C-fibres to peripheral electrical stimulation were inhibited to a greater extent, which was incidentally of greater significance in the neuropathic animals compared to the normal animals ($p<0.01$ and $p<0.05$ respectively). However, the magnitude of reduced activity is comparable for responses to high threshold natural stimuli (figs 4.3b-c and 4.5b-c). Thus, in the neuropathic state, descending facilitations could affect inputs to an increased number of cells, as well as inputs to individual cells such as small diameter primary afferents. It is not possible to rule out alternative mechanisms for the observed reduction in neuronal responses (there may additionally be post-synaptic effects on inhibitory or excitatory interneurones that lie pre-synaptic to the neurone being recorded from), yet several groups have previously concluded that descending neurones positively engage peripheral afferent fibres to increase spinal cord activity; Capsaicin-evoked neurotransmitter release from primary afferent fibres following spinal nerve ligation is lost in animals pre-treated with dermorphin-saporin (a toxic conjugate that ablates the μ-opioid receptor expressing facilitatory neurones in the brainstem) and in animals with DLF lesions (Gardell et al., 2003).

Previous work from our group has shown that the serotonergic system mediates at least some of the descending facilitatory effects since depletion of endogenous spinal 5HT leads to reductions in evoked responses of deep dorsal horn neurones in normal animals, and attenuates behavioural hypersensitivities to mechanical and cold stimuli after nerve injury (Rahman et al., 2006). Together these studies demonstrate the important role of the descending facilitatory system in the modulation of peripheral input which likely acts, at least in part, via permissive actions on spinal $5HT_3$ receptors located on the dorsal horn terminals of primary afferent fibres (Suzuki et al., 2002b). Nociceptive information arriving along these fibres ascends via superficial spinal projection neurones to the parabrachial area of the brain to precipitate supraspinal neoplastic changes that further facilitate, via

descending neurones in the RVM, incoming inputs in the deep dorsal horn, potentially resulting in an enhanced pain state. Dissection of the loop to establish the contribution of its constituent parts to neuropathic pain has already proved successful since site-specific ablation of NK1 receptor expressing lamina I neurones results in attenuated pain behaviours in animal models of nerve injury, accompanied by electrophysiological changes at the level of the spinal cord. These changes can be reproduced in control animals by intrathecal application of the $5HT_3$ receptor antagonist ondansetron (Suzuki et al., 2002b).

Within both populations of animals there were examples of lignocaine causing an *increase* in spinal neuronal activity following its injection into the RVM. Lignocaine would presumably not discriminate between cell types and fibres of passage within its diffusible injection area, thus these results may reflect a block of descending inhibitory cells, or cells driving these purported inhibitions, the aforementioned Off cells. In the relevant normal animals, post-discharge increased markedly, whilst the C-fibre responses increased to a lesser, albeit significant extent. Responses to brush and low-threshold mechanical stimulation increased more than the responses to high-threshold stimulation (which did not significantly increase post-lignocaine injection). In contrast to what has been suggested regarding descending facilitatory cells having a large pre-synaptic influence, descending inhibitory controls may have a predominant post-synaptic effect. Lignocaine's inability to significantly alter responses to high-threshold stimuli in this set of experiments could be because responses had reached a ceiling, implying that there was essentially no room for escalation in the event of disinhibition (i.e. inhibition of inhibitory cells becomes meaningless when responses are high). The variable response, either inhibition or facilitation following lignocaine's injection into the RVM suggests that On and Off cell firing is not reciprocal and mutually exclusive as originally thought, and that instead both types of cell can be active (with different degrees of influence) at any given time. This conclusion has similarly been drawn from studies that showed there is an increase in both the On cell discharge and number of Off cells recorded from in awake arthritic rats (Montagne-Clavel and Oliveras, 1994), and that pharmacological injection of agents into the RVM can recruit both classes of cells (see Section 4.1.3 above). Descending facilitations and inhibitions can therefore be triggered

simultaneously, with each potentially dominating activity in different areas of the body (for a review see Gebhart, 2004).

The alternative effects of lignocaine could be the result of divergent projections of facilitatory and inhibitory cells to different dorsal horn neurones. If a given neurone receives more synaptic input from facilitatory cells than inhibitory cells, then interrupting this input will lead to a reduction in the neurone's responses. Increased spinal responses would alternatively result if the cell being recorded from receives a greater input from inhibitory cells. Notwithstanding this, my results advocate a predominant descending facilitatory influence in animals unchallenged by pathological nociceptive inputs and enhancements of this facilitation in animals with experimental neuropathic pain. It has been shown that similar doses of intra-RVM lignocaine attenuate behavioural withdrawal responses to mechanical stimuli in neuropathic rats (Pertovaara et al., 1996). An important addition in our electrophysiology study is that responses to *both* threshold and supra-threshold stimuli are tested. Indeed, intra-RVM lignocaine exerted little influence over dorsal horn neuronal responses to low-intensity stimuli in normal animals, yet inhibited such responses in neuropathic animals. Furthermore, electrophysiology results from nerve-injured animals, recorded 14 or more days after ligation surgery, substantiate behavioural data showing the necessary contribution of the descending facilitatory system during the maintenance phase of experimental neuropathic pain (Burgess, 2002).

Neuronal transmission in different classes of axons, dendrites and cell bodies, as well as synaptic transmission between cells, are all susceptible to block by 0.8μl of 2% lignocaine (Fink and Cairns, 1984). In addition, as mentioned above, focal infusion of lignocaine will also block fibres of passage within the RVM. Deactivation of these travelling fibres could possibly account for the observed dorsal horn responses seen after injection of lignocaine into the RVM. This possibility does not however challenge my conviction that anaesthetic block reveals a tonic facilitatory influence, since the fibres traversing this area of the brainstem largely come from the PAG, which also gives rise to On and Off cells that have a role in spinal modulation and sensory determination (Heinricher et al., 1987).

There are various factors relating to this study that warrant some consideration so that they can be eliminated as potentially confounding factors or nuisance variables. The first of these relates to the general anaesthetic used since all rats used in these electrophysiology studies were anaesthetised with halothane to the point that they were areflexive (as measured by PWD to noxious pinch). RVM neurones are unquestionably modulated by anaesthetics, and arousal in general, yet On and Off cells are equally identifiable in unanaesthetised rats as they are in rats anaesthetised with ketamine, isoflurane and halothane (Clarke et al., 1994). Moreover, given that previous *in vivo* electrophysiology studies have identified the descending modulatory system and have shown that it is amenable to pharmacological manipulation (Suzuki et al., 2002b, Rahman et al., 2004), and that parallels can be drawn between electrophysiological and behavioural results in anaesthetised and unanaesthetised rats respectively (Rahman et al., 2006), there is a strong argument against a qualitative shift in the descending modulatory framework in the presence of anaesthesia. Since the anaesthetic level remained constant within and between the two populations of rats, changes were observed in a nearly constant system, the only controllable variable between the groups being nerve damage.

A second point relates to whether the observed spinal responses were due to specific pharmacological actions of lignocaine, or whether they were the result of physical disruptions of central neuronal activity in the injected area. Whilst I did not investigate this myself, others have shown that inert brainstem injections have no outward effect on responses, (Grossman and Stumpf, 1969). It is also unlikely that the effects observed in my studies were due to diffusion of lignocaine to adjacent structures, since not only is the volume of spread quite minimal (Martin, 1991), but misplaced injections in surrounding areas (see Figure 4.6) produced no discernible changes in electrophysiology.

Thirdly, suggestions that lignocaine may be acting at loci other than voltage-gated Na^+ channels to produce the given effects (for example it has been proposed that lignocaine may have 2nd messenger blocking effects which lead to long-term switching of neuronal function (Tomoda et al., 1990, Onozuka et al., 1993)) can be discounted since the concentration of lignocaine used in my study was lower than that which is required to produce these purported effects.

Finally, it is interesting to note that contrary to the results obtained in Chapter 3, which detailed the characterisation of the SNL model, the mean of the pre-drug control Aδ-fibre responses of the SNL animals in the present study is smaller than the mean of the pre-drug control Aδ-fibre responses of the normal animals (compare 97±13 with 62±25). Whilst this difference in itself is not statistically significant, it opposes the situation previously described whereby these values were 71±8 with 98±6 respectively (a difference that was statistically significant). Moreover, in the present study, the C-fibre responses evoked by electrical stimulation were significantly greater in the SNL rats relative to the normal rats ($P<0.05$), which was not identified in the characterisation study. Given that the data from this and the previous study are ostensibly from the same type of experiment in two different cohorts of rats, reasons underlying this discrepancy are not clear since many factors remained constant (including the breed of animal) between the experiments. However, one difference between the studies is the number of neurones tested, with the number sampled in the current drug study being far smaller than the number sampled in the characterisation study (for example, with respect to SNL rats these numbers were 26 and 66 respectively) which may at least partly account for the difference seen.

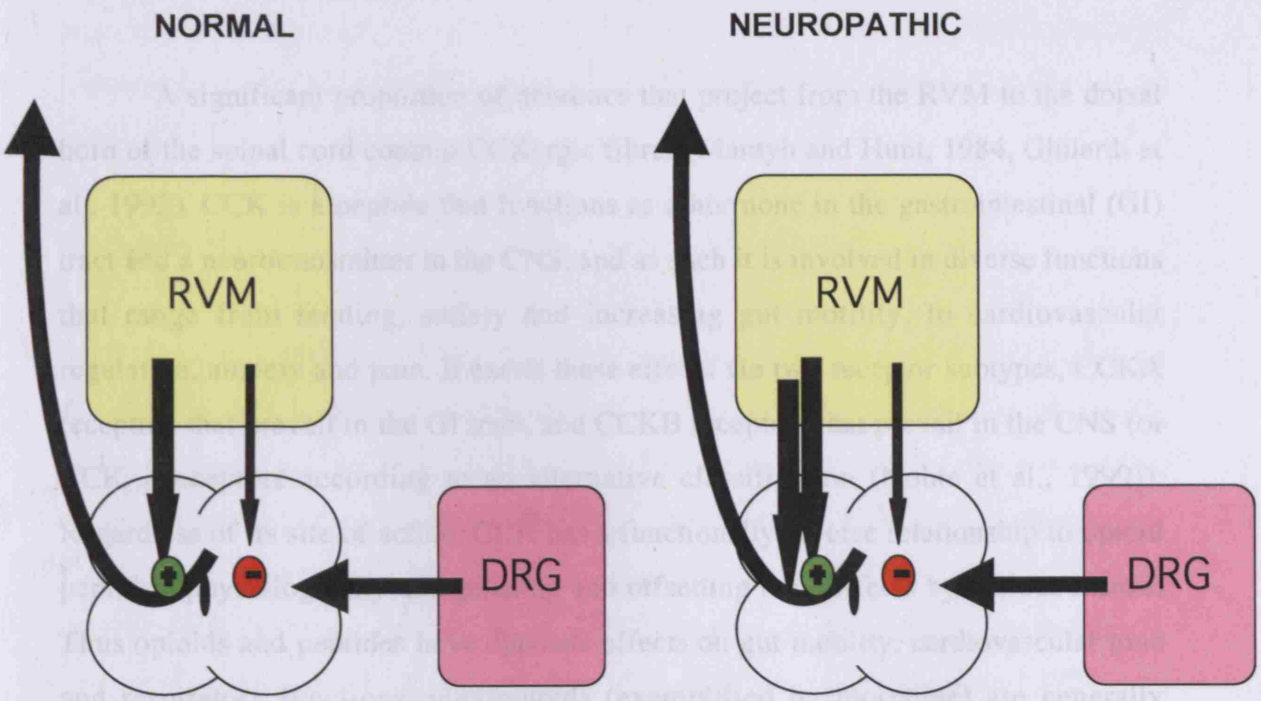
Lignocaine is used to treat various neuropathic pain states in humans, and is usually administered in the form of a 5% topical patch (see Table 1.3). It has been suggested that some of lignocaine's analgesic actions may occur independently of actions at peripheral nerve fibres (Chabal et al., 1989). In patients with painful diabetic neuropathy, systemic lignocaine can selectively reduce C-fibre-evoked polysynaptic reflexes (Bach et al., 1990b), which suggests that some effects are centrally mediated. In line with this, lignocaine's lipid: water diffusion co-efficient means that it quickly passes through the blood-brain-barrier to access the CNS upon systemic delivery. Given that spontaneous activities in damaged and adjacent peripheral nerves decline rapidly within a week of injury, lignocaine's preferential action in the CNS as opposed to the PNS could reflect greater activity at spinal and supraspinal structures, and thus a greater susceptibility to block than peripheral nerves. Moreover, the switching interplay between peripheral and central mechanisms of neuropathic pain may reasonably account for the multiple phases of relief (underscored by mechanistic differences) that a single infusion of lignocaine brings to SNL animals (Araujo et al., 2003). Lignocaine's preferential central action goes some

way towards explaining how systemic doses partially or totally relieve neuropathic pain symptoms in patients without blocking motor or sensory function (Boas et al., 1982).

The results in this study are relevant to animals with neuropathic pain, yet parallels can be drawn with models of inflammatory pain given the evidence for the role of medullary pain facilitating neurones in inflammation-induced secondary hyperalgesia (Kincaid et al., 2005) and the changing profiles and phenotypic switches in the RVM that accompany peripheral inflammation (Miki et al., 2002, Imbe et al., 2005, Imbe et al., 2007). Using *in vivo* electrophysiology techniques, recording from dorsal horn wide-dynamic-range neurones (as in the present study) the supraspinal contribution to mustard-oil induced changes in neuronal responses have been demonstrated, again through the injection of lignocaine (1μl, 4% w/w) into the RVM (Pertovaara, 1998). Our group has shown however that unlike the situation during neuropathy (Suzuki et al., 2002b), descending serotonergic facilitation mediated through spinal $5HT_3$ receptors is unaltered following carageenan inflammation (Rahman et al., 2004). It would therefore be interesting to deduce alternative mechanisms responsible for the observed alterations in spinal neuronal responses after inflammation.

In summary, I present electrophysiological evidence for the existence of RVM-mediated descending controls that operate in both normal animals and animals with spinal nerve injuries. My results, which show a predominant inhibitory effect of intra-RVM lignocaine on the electrical- and high threshold natural-evoked responses of deep dorsal horn neurones in normal animals suggest that descending facilitatory influences prevail in these animals. Moreover, the increase in the proportion of experiments where this spinal reduction was seen in the SNL animals, as well as the magnitude of the reduction, points to an increase in the descending facilitatory drive after nerve injury.

Figure 4.7 *Summary diagram showing that descending modulations from the RVM weight in favour of facilitations in the normal state, and increase after nerve injury.*



5. CHOLECYSTOKININ (CCK) IN THE RVM

5.1 CCK AS A HORMONE AND NEUROTRANSMITTER

A significant proportion of neurones that project from the RVM to the dorsal horn of the spinal cord contain CCKergic fibres (Mantyh and Hunt, 1984, Ghilardi et al., 1992). CCK is a peptide that functions as a hormone in the gastrointestinal (GI) tract and a neurotransmitter in the CNS, and as such it is involved in diverse functions that range from feeding, satiety and increasing gut motility, to cardiovascular regulation, anxiety and pain. It exerts these effects via two receptor subtypes, CCKA receptors that prevail in the GI tract, and CCKB receptors that prevail in the CNS (or $CCK_{1/2}$ receptors according to an alternative classification (Noble et al., 1999)). Regardless of its site of action, CCK has a functionally inverse relationship to opioid peptides, physiologically antagonising and offsetting their effects by various means. Thus opioids and peptides have opposite effects on gut motility, cardiovascular tone and respiratory functions, plus opioids (exemplified by morphine) are generally amnesic, analgesic and anxiolytic, whilst CCK is mnemonic, algesic and anxiogenic.

5.1.2 THE NOCEBO EFFECT ("I SHALL HARM")

CCK's ability to induce fear and panic was proposed to underlie the nocebo response to pain (for a review see (Benoliel et al., 1991)). In contrast to the placebo effect (discussed in Section 1.3.3.1), the nocebo effect refers to the worsening of symptoms that occurs as a result of expecting a negative outcome (akin to catastrophising); negative verbal suggestions instil a negative psychosocial setting that induces anticipatory anxiety and negative outcome, which, in the context of pain, and in it's extreme, can change the nature of an agent so that it becomes hyperalgesic instead of analgesic (Dworkin et al., 1983). The nocebo effect activates the central CCKergic system, which facilitates nociceptive transmission and amplifies pain, an effect that can be reversed by CCKB receptor antagonists (Benedetti et al., 2006). As in Chapter 4, this illustrates the powerful top-down control of sensory input that shapes pain perception, and exemplifies the important role of cognition and affect in therapeutic outcome. The close relationship between nocebo hyperalgesia and anxiety was examined in a group of post-operative patients who each received an inert

substance that they were told would be painful (Benedetti et al., 1997). Proglumide, a CCK receptor antagonist that is not analgesic *per se*, was shown to reduce the nocebo effect in a dose-dependent manner, yet it did not do this by blocking anticipatory anxiety of the impending pain. Thus, it seems that the CCK system can alter painful outcome by mechanisms that are independent of its ability to influence psychological state.

5.1.3 CCK8

In the CNS, CCK is found as a carboxy-tailed octapeptide (CCK8), and it's distribution within spinal and supraspinal areas matches the distribution of opioids and their receptors, which themselves widely overlap with nociceptive modulating sites (Stengaard-Pedersen and Larsson, 1981, Baber et al., 1989). This morphological co-localisation hints at the counter-modulatory interactions of these different peptides in nociceptive processing, and indeed, CCK8 suppresses the anti-nociceptive effects produced by endogenous opioids (Itoh et al., 1982). Therefore the central CCKergic system represents an anti-opioid system that functions as a physiological feedback, influencing the balance between facilitation and inhibition of excitatory input, thus contributing to homeostatic control of nociceptive transmission (Rothman, 1992).

It is not entirely clear how CCK8 attenuates opioid analgesia, but several mechanisms have been proposed. These include reports that activation of CCKB receptors by CCK8 reduces the binding affinity of opioids to their receptors (Wang et al., 1989), and also the hypothesis that CCK8 acts as a competitive antagonist at opioid receptors, directly preventing opioids from binding and eliciting their inhibitory effects (yet whilst one study has demonstrated the modest and variable capacity of different CCK receptor antagonists to bind to opioid receptors(Gaudreau et al., 1990), there is no evidence that CCK itself can bind to opioid receptors). It seems that a more plausible explanation for the counter effects of CCK with respect to opioids involves the 2nd messenger signalling system and intracellular Ca^{2+} concentrations. CCK, like morphine (for example) binds to a GPCR, yet unlike the MOR, the CCK receptor is linked to a G_q protein and is therefore related to increased neurotransmitter release via activation of intracellular signalling enzymes (IP3 and DAG) and increased Ca^{2+} conductance. This directly opposes the morphine-G_i-protein

linked inhibition of neurotransmitter release. CCKB receptors and MORs co-localise at the central terminals of primary afferent fibres that synapse in laminae I and II of the dorsal horn (and incidentally, interneurones in these superficial laminae precede descending neurones as the main source of spinal CCK). Spinal analgesia by exogenous or endogenous opioids occurs predominantly by a presynaptic suppression of glutamate and SP release from these central terminals (Dickenson, 1991) (Yaksh et al., 1980). This somehow leads to increased release of CCK8 in the spinal cord (Gustafsson et al., 1999), which is thought to feedback and facilitate excitatory transmitter release from these peripheral afferents, thereby opposing the inhibitory strength of morphine. SP, and NK1 receptors in particular, have been singled out in CCK's effects since intrathecal administration of a NK1 receptor antagonist attenuates CCK8's pronociceptive influence (in the presence of morphine analgesia) (Fukazawa et al., 2007). Thus release of spinal CCK8 and subsequent release of SP and activation of NK1 receptor-expressing projection neurones may be a key way in which CCK attenuates morphine analgesia and promotes nociception.

Additionally or alternatively, CCK8 may mediate its effects via the GABAergic system (Acosta, 2001, Brack and Lovick, 2007). GABA released from inhibitory interneurones suppresses the spinal release of excitatory neurotransmitters from primary afferent neurones by presynaptic inhibition (Barber et al., 1978), and it is thought that CCK8 may target this inhibition; as described above, CCK8 activates G_q proteins upon binding to its receptor, which, via a cascade of intracellular reactions, activates the enzyme PKC. This in turn phosphorylates $GABA_A$ receptors to restrict their function (Chen et al., 1990). Thus CCK8 may suppress the responses of DRG neurones to GABA, thereby negating or finely balancing the analgesic actions of opioids so that they remain within physiologically- and state-determined limits (Kolaj and Randic, 1996).

5.1.4 SUPRASPINAL ACTIONS OF CCK8

The anti-opioid actions of CCK8 are not site specific since its injection into the RVM (like its intrathecal administration) can displace the *spinal*-morphine dose-response curve to the right (Xie et al., 2005). Other studies have demonstrated the localised interaction of CCK8 and opioids in supraspinal areas (Kovelowski et al.,

2000). CCK8 in the brainstem drives descending facilitations during continuous morphine exposure, with microdialysate fluid in rats showing an approximately 5-fold increase in basal levels of CCK8 within brainstem areas (relative to controls) during ongoing opioid infusion (Xie et al., 2005b). This surge in CCK8 is thought to represent one mechanism by which tolerance to morphine and the phenomenon of paradoxical pain occur (Ossipov et al., 2005).

CCK-immunoreactivity in the brainstem resides mainly within the RVM (Hokfelt et al., 1988), with actions also reported in the PAG (Liu et al., 1994) and the nACC (Kombian et al., 2004). Whilst some studies have concluded that CCK8 by itself has no bearing on nociceptive thresholds in the absence of opioid analgesia (Watkins et al., 1985, Wiesenfeld-Hallin and Duranti, 1987, Fukazawa et al., 2007), other studies have shown that injection of CCK8 into the RVM of naïve rats produces CCKB receptor-mediated hypersensitivities (Heinricher and Neubert, 2004, Xie et al., 2005). DLF lesions can reverse these, which suggests that descending facilitatory mechanisms are responsible. Indeed, focal infusion of 30ng CCK8 in normal animals increases On cell activity by both reducing the threshold at which their burst firing is triggered, and altering the patterned input such that the On cells are more likely to be active at any given time, producing a behaviourally-measurable hypersensitive state in the absence of any effect on Off cells (Heinricher and Neubert, 2004). A lower dose (10ng) of CCK8 in the RVM does not affect ongoing activity of any class of cell, yet blocks morphine-induced activation of Off cells (Heinricher et al., 2001a). Taken together, these results suggest that the pro-nociceptive and anti-opioid effects of CCK8 are mediated by distinct neural elements (On and Off cells respectively) that may operate independently of one another, or, as is more likely the case, in parallel.

Given that CCK8 can exert post-synaptic effects in the CNS, direct activation of facilitatory On cells probably explains its pronociceptive actions. However, since opioid activation of Off cells must occurs by indirect means (see Section 1.3.3.3), CCK's anti-opioid effects (i.e. interference of Off cell activation) must somehow occur pre-synaptically. As explained above, the excitatory effects of CCK8 may occur by interactions with the GABAergic system, an effect that has additionally been described in the PAG (Brack and Lovick, 2007) and the nACC (Kombian et al., 2004), as well as in higher centres of the brain such as the hippocampus where CCK

acts via CCKB receptors to decrease the firing frequency of GABA-containing neurones (Deng and Lei, 2006). Dependence on the GABAergic system explains how CCK-induced neuronal excitability in the dorsal PAG varies according to the different stages of the oestrous cycle (Brack and Lovick, 2007): changes in the functional properties of GABAergic neurones in the dPAG have been suggested to underlie the varying responsiveness of neurones in this area to intravenous or iontophoretic injection of a CCKB receptor agonist. Perhaps the ability of this agonist to alter the firing rate of GABAergic neurones in this area is mechanistically analogous to the ability of CCK8 (and associated agonists) to alter the firing rate of GABAergic neurones in the RVM that lie presynaptic to Off cells.

5.1.5 CCK AND NEUROPATHIC PAIN

So far I have only described CCK's actions in the naïve, or morphine-exposed setting, yet this peptide also plays a major role in chronic pain, with different effects (or CCK-opioid effects) in different pain states. Thus, in inflammatory pain, the potency of intrathecal morphine increases (relative to the normal state) and is unaffected by CCKB receptor antagonists, which suggests an inflammation-induced reduction in CCK activity (Stanfa and Dickenson, 1995). On the contrary, in neuropathic pain states, morphine's potency is reduced (Portenoy et al., 1990). Together with observations that nerve-injured mice lacking the CCKB receptor do not develop behavioural hypersensitivities (and have up-regulated opioid systems) (Pommier et al., 2002, Kurrikoff et al., 2004), this suggests an increase in CCK activity consequent to nerve injury, and indeed, animal models confirm that elevated levels of CCK mRNA in the DRG accompany peripheral nerve injury (Xu et al., 1993). Furthermore, injecting a specific CCKB receptor antagonist into the RVM reverses SNL-induced behavioural hypersensitivities and restores the potency and efficacy of PAG-injected morphine (Kovelowski et al., 2000). Therefore in addition to CCK plasticity at the spinal level (Stanfa et al., 1994), supraspinal changes may also explain the relatively poor efficacy of opioids in clinical neuropathic pain. It seems that maintained descending facilitations depend on supraspinal CCK release (that may be driven by a barrage of afferent input from the periphery), which exerts pronociceptive effects per se and compromises the analgesic efficacy of endogenous and exogenous opioids.

Given that CCK is a critical component of the descending facilitatory pathway, I intend to inject CCK8 into the RVM of naïve and nerve-injured animals to provide novel information about the effects of this peptide on the evoked responses of dorsal horn neurones to a range of stimuli in normal and pathophysiological states, and in a similar way to my studies with lignocaine (Chapter 4), I aim to provide neuronal correlates to existing behavioural data.

5.2 METHODS

5.2.1 SNL SURGERY, BEHAVIOURAL TESTING AND IN VIVO ELECTROPHYSIOLOGY

As described in Section 4.2.1, except 23 naïve animals, and 15 animals that had received nerve injuries 14-17 days previously were used in the electrophysiology study.

5.2.2 DRUG ADMINISTRATION

The protocol for drug administration was exactly as described in Section 4.2.2, except 1μl of 50ng CCK8 was injected into the RVM following stabilisation of a neurone's response.

5.2.3 DATA ANALYSIS

Behavioural data are presented as mean±SEM paw withdrawal response to von Frey 6g and 8g on each post-operative day tested (ipsilateral paw versus contralateral paw). Statistical significance was calculated using non-parametric Wilcoxon matched pairs tests.

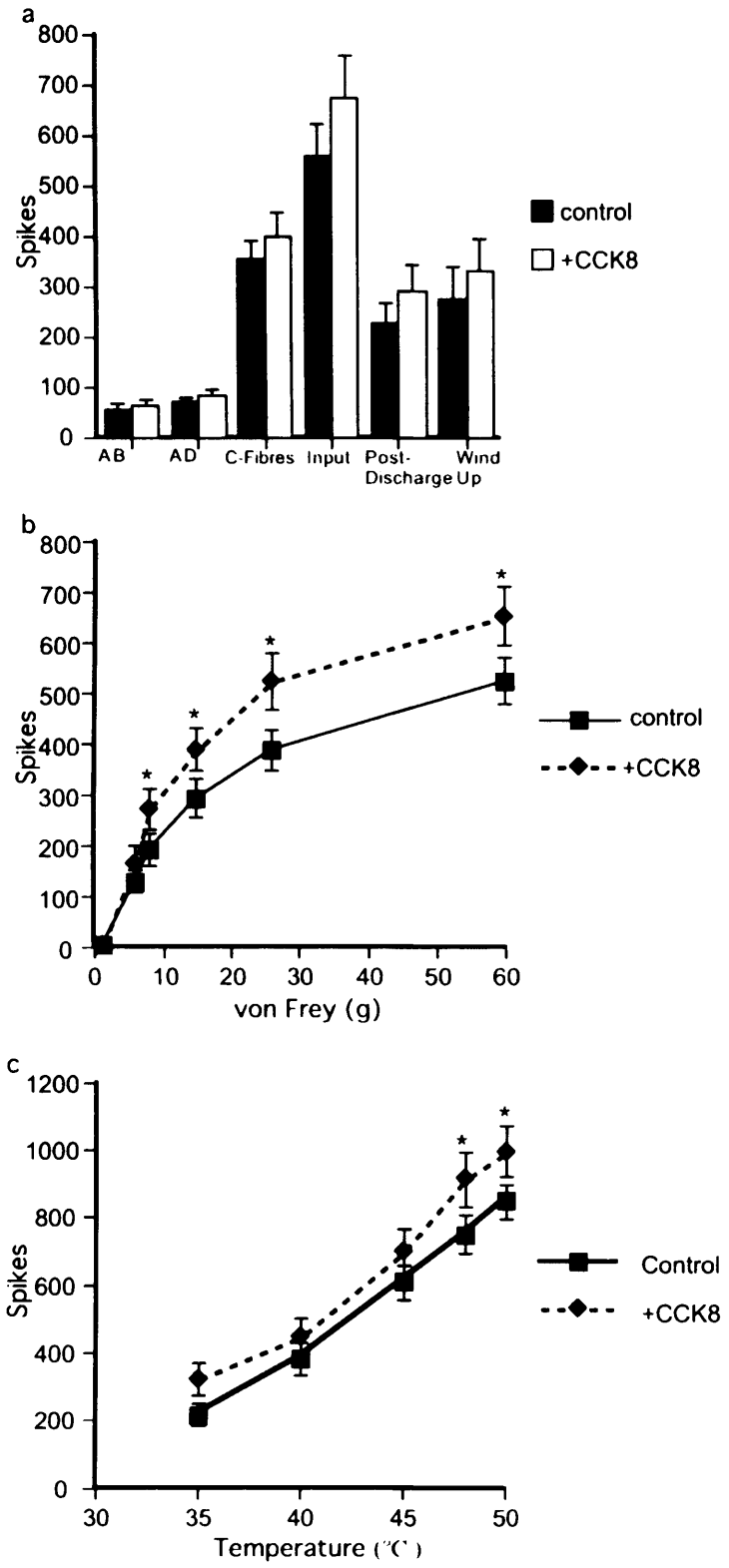
Electrophysiological raw data are presented as mean±SEM response (number of spikes evoked by a given stimulus) for averaged pre-drug controls, and the effects of CCK8 on the electrically- and naturally-evoked responses are presented as maximal change from the averaged pre-drug control for each measure. The significance of drug effects was calculated using one-way ANOVA and Dunnett's post-hoc tests for electrical responses, whilst two-way ANOVA with Bonferroni multiple comparisons tests were used for responses to mechanical and thermal stimuli.

5.3 RESULTS

5.3.1 THE EFFECTS OF INTRA-RVM CCK8 IN NORMAL ANIMALS

Microinjection of 50ng CCK8 into the RVM of normal animals did not significantly alter the responses of dorsal horn neurones to electrical stimulation of the hindpaw (Figure 5.1a). However, responses to vFs 8g, 15g, 26g and 60g were significantly elevated following CCK8 injection (Figure 5.1b), as were responses to 48°C and 50°C (Figure 5.1c).

Figure 5.1 *Responses of dorsal horn neurones to electrical (a), mechanical (b) and thermal (c) stimulation of the hindpaw in normal animals (n=23), both before (control) and after the injection of CCK8 into the RVM.*



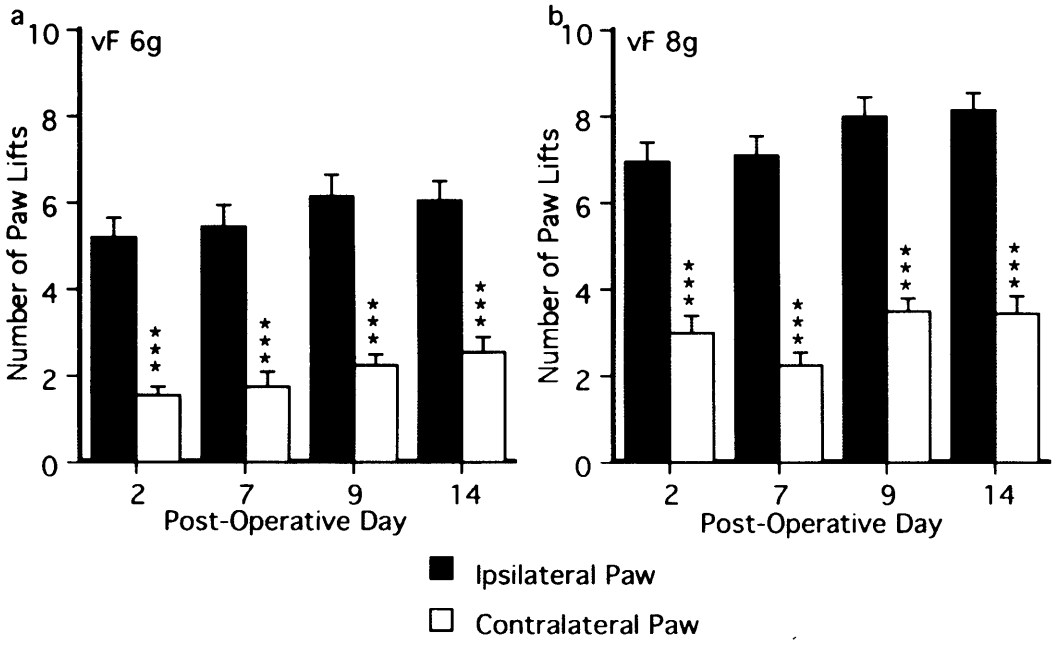
Intra-RVM CCK8 increased dorsal horn neuronal responses to mechanical stimulation (b) and noxious thermal stimulation (c) in the 23 naïve animals tested. (*P<0.05).

To interpret and analyse results from these studies, as well as corresponding results from SNL animals (below), I looked at the biggest change in response that RVM-CCK8 produced on each of the measured variables, for each animal over the 120-minute testing period (therefore I did not look at the time-course of effect and statistically examine responses at each time-point as I did with lignocaine in Chapter 4). The results and statistical significance (where present) therefore represent the data as a whole, with no distinction between each different time point (N.B. the results are from experiments with histologically-verified injection sites within the RVM). As an aside, inspection of the raw data shows that within this normal group, there were 7 neurones (from 7 rats) that had highly elevated responses to mechanical and thermal stimuli as listed, as well as to electrical stimuli, specifically C-fibre-responses, input and post-discharge, 20 and 40 minutes after the injection of CCK8 into the RVM and so I wondered whether there was anything physiologically different about these neurones compared to the other 16 cells in this group; C-fibre thresholds and depth within the spinal cord were not notably different, yet the pre-drug responsiveness of neurones to mechanical and electrical stimuli within this sub-group tended to be lower (although t-tests do not confirm this to be statistically relevant).

5.3.2 DEVLOPMENT OF BEHAVIOURAL HYPERSENSITIVITIES FOLLOWING SNL SURGERY

As in Sections 3.3.1 and 4.3.2, SNL surgery was followed by rapid development of behavioural hypersensitivities, as represented in Figure 5.2 below by ipsilateral v. contralateral number of paw lifts in response to vFs 6g and 8g.

Figure 5.2 *The number of ipsilateral paw lifts in response to vF 6g (a) and vF 8g (b) were significantly higher than the number of contralateral paw lifts on days 2, 7, 9 and 14 after SNL surgery.*

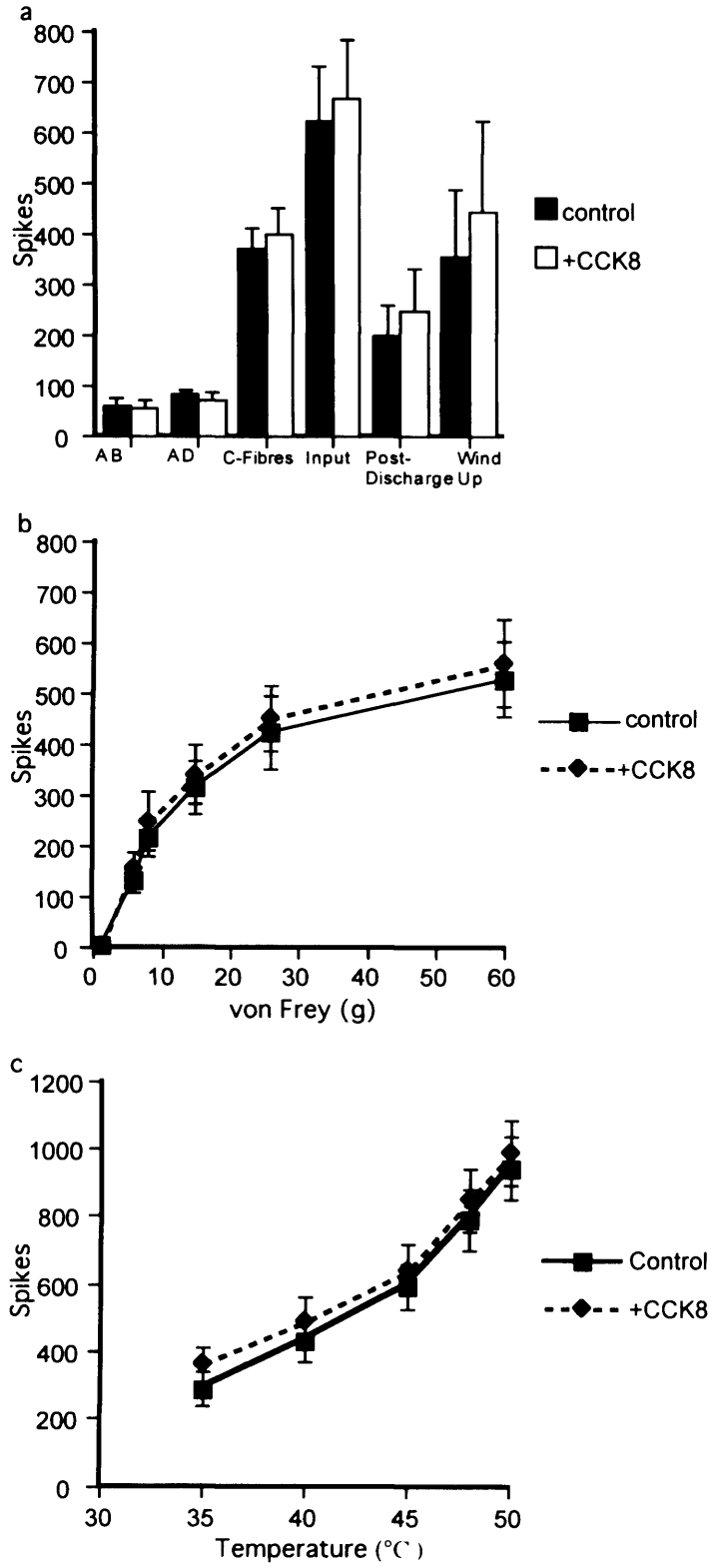


Behavioural hypersensitivity to vF6g (a) and vF8g (b) as well as vF 1g, 15g and acetone (data not shown) developed rapidly in rats following SNL surgery (n=15). Hindpaw withdrawal responses increased significantly in the paw ipsilateral to the side of nerve injury (filled bar) relative to the contralateral paw (open bar) at post-operative days 2, 7, 9 and 14 (***P<0.001).

5.3.3 THE EFFECTS OF INTRA-RVM CCK8 IN SNL ANIMALS

Electrophysiological recordings were made on either post-operative day 14, 15, 16 or 17 when behavioural hypersensitivities were stable. Results show that injection of CCK8 into the RVM did not significantly alter the evoked responses of dorsal horn neurones to any of the applied stimuli, electrical or otherwise (Figure 5.3).

Figure 5.3 *Responses of dorsal horn neurones to electrical (a), mechanical (b) and thermal (c) stimulation of the hindpaw in SNL animals (n=15), both before (control) and after the injection of CCK8 into the RVM.*



Intra-RVM CCK8 did not alter dorsal horn neuronal responses to any of the applied peripheral stimuli in SNL animals.

5.4 DISCUSSION

The concept that descending influences from the brainstem can modulate the transmission of sensory signals from the periphery, and that the RVM in particular is the source of the descending tracts, has been discussed in previous chapters. This current chapter continues with this theme since I have presented data showing the spinal effects of intra-RVM CCK8 in both normal and nerve-injured animals: By recording the evoked-responses of dorsal horn neurones to both threshold and supra-threshold stimuli, I have shown that 50ng of CCK8 injected into the RVM has a greater effect in normal animals than it does in SNL animals. Indeed, whilst there was a slight tendency for responses to electrical stimuli and coding of mechanical and thermal stimuli to increase after CCK8 injection in SNL animals, elevated responses did not reach statistical significance.

Not long after CCK was identified in the CNS, the RVM was identified as a source of both CCK and opioid peptides, and it was further shown that receptors for CCK and morphine co-localised together within the brainstem, particularly in areas associated with pain modulation (Mantyh and Hunt, 1984)(Bowker and Dilts, 1988, Baber et al., 1989). Given the close neuroanatomical distribution of the opioid and CCK systems, it became apparent that these peptides were linked, albeit antagonistically, in their modulation of certain physiological settings. What wasn't so clear, and still isn't, is whether this link is inextricable or whether the two systems can operate independently and just incidentally influence one another. Opinions vary on whether CCK8's 'pro-nociceptive' actions are a product of its anti-opioid effects, or inversely, whether the 'anti-opiod' effects of CCK8 are merely the result of enhanced nociception (which inevitably leads to higher opioid requirements). This latter view at least acknowledges an independent role for CCK8 in nociceptive transmission, and thus a role that is not bound to a functioning opioid system. Deletion of the gene that encodes the CCKB receptor (CCKB-/-) yields knock-out mice that display significantly reduced mechanical sensitivities (i.e. hyposensitivities) compared to wild-type (WT) littermates, an effect that can be dose-dependently mimicked and induced in these littermates by a CCKB receptor antagonist (Kurrikoff et al., 2004). This seems to suggest that CCK *per se* contributes to the regulation of mechanical sensitivities, and by inference, nociceptive thresholds too, which argues in favour of

an independent role for CCK8 in nociceptive processing. However, this premature conclusion is confounded by subsequent results, which showed that mechanical sensitivities in the CCKB-/- mice could be reversed to 'normal' levels shown by WT mice by naloxone injection (Kurrikoff et al., 2004). Thus it seems that compensatory mechanisms arising from the invalidated CCKB receptors were responsible for the observed effects. One of these mechanisms is an up-regulated opioid system (Pommier et al., 2002), which itself can increase the thresholds for nociceptive stimuli (Veraksits et al., 2003).

In the first set of studies detailed herein, I have shown that local infusion of CCK8 into the RVMs of animals with no pre-existing pain state increased dorsal horn neuronal responses to innocuous and noxious mechanical stimuli, as well as responses to noxious thermal stimuli, which is in line with previous behavioural data from naïve animals (Kovelowski et al., 2000, Heinricher and Neubert, 2004). Since the combined microinjection/recording technique that I used meant that I could not have easily administered additional drugs into the RVM after CCK8 injection, I did not directly address the question of opioid involvement in theses effects (i.e. were the increased responses due to parallel reductions in endogenous opioid actions, either at the spinal or supraspinal level?), yet the injected dose was chosen since such a high dose (as opposed to a low dose of 10ng) is known to selectively activate On cells (Heinricher and Neubert, 2004). It could be said that this 'activation' is a result of reduced morphine inhibition of On cells (since cells which appear similar to On cells *in vivo* express MORs), yet since this dose did not interfere with the firing of Off cells, it seems that it did not alter the opioid system.

Despite descending facilitations playing a constitutive role in spinal sensory processing in normal animals, the contribution of CCK8 to this transmission is thought to be modest (Coudore-Civiale et al., 2000), with just a minimal amount of CCK8 being released in the CNS during homeostasis (Tang et al., 1984, Benoliel et al., 1991). However, disturbances in the system at cardiovascular, respiratory, psychological or sensory levels for example can cause the release of CCK8 within the CNS, which works independently, or in concert or against other peptides to re-establish the normal state. Sometimes this can have its drawbacks, and in particular in neuropathic pain the upregulated role of CCK is particularly damning since not only

does it enhance nociceptive transmission, but it compromises responses to opioids and may promote opioid-induced paradoxical pain, possibly in areas unrelated to the initial pain complaint (De Conno et al., 1991, Doverty et al., 2001). In the pursuit of homeostasis, CCK8 levels increase after nerve damage to offset any potential sensory loss. This therefore enables a barrage of largely uninhibited afferent input to access the spinal cord and trigger spinal sensitisation, and in a feed-forward way this elicits neuroplastic changes in supraspinal areas so that descending facilitatory controls further enhance the sensory tone in the spinal cord, an effect that is additionally driven by CCK8's actions in the RVM (Kovelowski et al., 2000).

To emphasise the importance of the CCKergic system in neuropathic pain, studies have shown that deletion of the CCKB receptor gene as described above abolishes the development of behavioural hypersensitivities in mice following ligation of the sciatic nerve (Kurrikoff et al., 2004), whilst in SNL rats, the injection of a specific CCKB receptor antagonist into the RVM reverses established behavioural hypersensitivities (Kovelowski et al., 2000). In my study, 50ng of CCK8 injected into the RVM did not significantly alter neuronal responses to any of the applied peripheral stimuli in nerve-injured rats. Given CCK8's role in neuropathic pain and the fact that this dose did alter neuronal responses in normal animals, it could be that the supraspinal CCK system had saturated in the SNL rats. This could either be due to full occupancy of RVM CCKB receptors by endogenous CCK8, which inevitably means that surplus CCK8 in the form of an exogenous injection literally has no room to cause an effect, or it could be that the maximal effect of receptor stimulation (which may occur at low or incomplete receptor occupancy) had been reached. In terms of facilitatory On cells, this effect is an increase in their firing rate. If the RVM-CCK8 concentration is high enough (i.e. as a result of SNL-induced release), this may result in an overall increase in ongoing On cell activity in the absence of characteristic burst firing, as has been seen following the injection of 30ng of CCK8 into the RVMs of naïve animals (Heinricher and Neubert, 2004). Therefore CCK8 that is released as a consequence of nerve injury may have maximally increased the ongoing activity and patterned input of On cells to the extent that additional CCK8, in the form of an injection, would have little effect on measured responses, with the probability of the On cells being active at any given time remaining constant.

That is not to say that the nociceptive and pain-modulating effects of CCK plateau in line with its maximal effect on On cell output, just that my results in SNL animals may reflect this. Indeed, the effects of central CCK are more far-reaching than this since its anti-opioid actions are thought to at least partly explain opioid tolerance whereby higher and higher doses of the opioid are required to elicit the same level of analgesic efficacy either clinically (Foley, 1995) or in animal studies (Ossipov et al., 2003, Suzuki and Dickenson, 2006). In addition, a state of hyperalgesia known as paradoxical pain that results from prolonged use of opioids (Yaksh et al., 1986) is thought to be CCK-mediated (Gardell et al., 2006). Further compounding the pain complaint is anxiety-induced hyperalgesia (Andre et al., 2005); the co-morbid presentation of anxiety and/or depression with neuropathic pain is underscored in both respects by an up-regulated CCK system (Campbell et al., 2003), thus each can affect and be affected by the other in a mutually reciprocal manner. Given the dual role of the opioid-CCK link in both the modulation of anxiety and nociception, a novel agent with central antagonist actions at CCKB receptors and simultaneous activity at mu-opioid receptors could be efficacious at multiple interacting levels (Hruby et al., 2003).

As an aside, it is interesting to note that activation of central serotonergic mechanisms by CCK8 is involved in the control of satiety and other GI functions (Stallone et al., 1989, Grignaschi et al., 1993); CCK8 released post-prandially excites serotonergic neurones (Boden et al., 1991) so that 5HT is released in the hypothalamic periventricular nuclei and supraoptic nuclei (Gershon and Erde, 1981). The number of parallels between the GI and sensory systems, particularly with respect to 5HT and CCK is striking. Firstly, 5HT and CCK influence the level of sensory gain in both systems, and in a similar way that aberrations in the nociceptive system can lead to hyper-phenomena whereby stimuli elicit exaggerated responses (allodynia and hyperalgesia as described in Section 1.2.1), which is partly the result of reduced sensory thresholds, an altered central 5HT system can reset the threshold of various gut receptors, including mechanoreceptors and CCK receptors so that normally 'innocuous' stimuli (gut distension, food in the stomach or nutrients in the small intestine for example) can trigger abnormal afferent input, analogous to that conveyed by A- and C-fibres during ongoing nociception, eventually leading to an altered GI function and distortion of GI perception (French et al., 2000, Feinle et al., 2001). Thus

just as abnormalities in CCK or 5HT pathways can perturb feeding and GI function, they can also affect normal sensory processing as described. Furthermore, in a similar way that emotions and cognitive state can influence pain, they can also influence feeding and associated physiological effects such as GI secretions and motility (Mayer, 2000, Monnikes et al., 2001). Indeed, stress and psychological factors may trigger a constellation of dyspeptic symptoms in patients, one of which is pain (Chua et al., 2006). Hence given the translational mechanisms between GI function and nociception, and CCK's presence in the RVM, an area that includes the serotonin-rich NRM, it could be that the serotonergic and CCKergic systems interact to alter nociceptive transmission and perception.

6. THE ROLE OF MEDULLARY MU-OPIOID RECEPTOR EXPRESSING NEURONES IN THE SPINAL PROCESSING OF SENSORY INFORMATION

6.1 RVM NEURONAL HETEROGENEITY

The previous chapter briefly described how the RVM, and in particular the actions of CCK within the RVM, at least partly mediates opioid-induced hyperalgesia and paradoxical pain. It has been suggested that the activity of On cells is enhanced during both these phenomena (Bederson et al., 1990), an idea that is supported by findings that opioid tolerance and withdrawal can be decreased by microinjection of lignocaine into the RVM (Deakin and Dostrovsky, 1978, Kaplan and Fields, 1991, Ossipov et al., 2005). The RVM's central role in opioid analgesia has been well-described (Fields et al., 1991, Fields, 2004) and neurones within this brainstem area, some of which project down to the spinal cord, can express multiple opioid receptor subtypes, with functionally distinct classes of cells bearing different receptor expression profiles (Marinelli et al., 2002). *In vitro* recordings from RVM neurones have shown that the vast majority (>90%) of spinally-projecting neurones (identified by previous retrograde tracer injections into the spinal cord) respond to opioid receptor agonists, with over half responding to μ-opioid receptor (MOR) agonists only, approximately a quarter responding to both MOR and κ-opioid receptor (KOR) agonists, and a smaller proportion responding to KOR agonists alone. Moreover, many of the μ-responsive spinally projecting neurones in the RVM stained positive for TPH, a marker of serotonin content, and conversely, many of the TPH+ spinally-projecting RVM neurones responded to MOR agonists. There were however also examples of non-serotonergic and/or non spinally-projecting RVM neurones that also responded to opioid receptor agonists (and MOR agonists in particular) which suggests that important roles exist for different classes of RVM neurones in the mediation of supraspinal opioid effects, an idea that has been confirmed by others, particularly with respect to morphine's antinociceptive actions (Marinelli et al., 2005, Winkler et al., 2006).

Since RVM On and Off cells are primarily classified according to changes in their evoked activity following noxious stimulation (Fields et al., 1983a, Fields and Heinricher, 1985b, Barbaro et al., 1986a), they cannot be identified in slice preparations. However, comparisons of behavioural and *in vitro* studies suggest that cells referred to as 'primary' cells *in vitro* appear similar to Off cells *in vivo*, and cells referred to as 'secondary' cells *in vitro* appear similar to On cells *in vivo* (Fields et al., 1983b, Fields et al., 1991, Heinricher et al., 1994). Thus *in vitro* slice recordings have shown that putative On cells are directly hyperpolarised and inhibited by μ-opioid receptor agonists, whilst putative Off cells receive μ-opioid sensitive GABAergic inputs (Pan et al., 1997). This fits with a model that suggests that facilitatory On cells are directly inhibited by morphine, and that inhibitory Off cells are indirectly activated by morphine (Ackley et al., 2001), which neatly explains central morphine-induced antinociception. Furthermore, as will be discussed later in this chapter, the fact that many TPH+ cells responded to MOR agonists lends support to the idea that serotonergic mechanisms are intrinsically part of the RVM's descending facilitatory output.

Despite being indirectly activated by MOR agonists, primary 'Off' cells are directly (and probably indirectly too) inhibited by KOR agonists (Pan et al., 1997). This explains how the administration of KOR agonists in the RVM attenuates the behavioural antinociception produced by MOR agonists administered either systemically or into the PAG (Pan et al., 1997). Juxtacellular recording and labelling techniques in conjunction with the tail flick test in behaving rats has shown that κ-opioid responsive Off cells typically stain positive for GAD67, a marker of GABA content (Winkler et al., 2006). Whilst it is true that GAD67 is present in all three classes of RVM neurones, On cells are least likely to stain positive for this enzyme, whilst Off cells are the most immunoreactive (Winkler et al., 2006). These findings, together with findings that many spinally-projecting RVM neurones express κ-opioid receptors (Marinelli et al., 2002), suggest that μ-activated Off cells send inhibitory projections down to the spinal cord to evoke antinociception.

A significant proportion of On cells project from the RVM to the spinal cord (Vanegas et al., 1984) and in particular the dorsal horn (Fields et al., 1995). Given that many spinally-projecting μ-opioid responsive neurones are serotonergic

(Marinelli et al., 2002), it is likely that some, but not necessarily all On cells use 5HT as a neurotransmitter, and in reverse that some, but not necessarily all serotonergic neurones are On cells. Indeed, in the anaesthetised rat, putative serotonergic neurones that were identified by antidromic conduction velocities showed excitatory responses to noxious sensory stimuli (Wessendorf and Anderson, 1983). There are however many claims to the contrary, with different groups suggesting for example that noxious-heat-responsive medullary serotonergic neurones are neither On or Off cells (Gao and Mason, 2000) and that serotonergic neurones are contained in one physiological cell class within the RVM (Potrebic et al., 1994).

Potential discrepancies between studies may be explained in terms of number of cells sampled, and in particular the sampled population not representing the whole population (an explanation which embraces the concept that the RVM serotonergic population is a pharmacologically, electrophysiologically and functionally heterogenous group of cells). Indeed, in one study which concluded that serotonergic cells in the brainstem are 'not strongly affected by noxious heat', which necessarily implies that they are not On or Off cells, just thirteen 5HT-ir cells were sampled, and of these thirteen cells, three were 'weakly excited by noxious heat' whilst a further two serotonergic cells were 'briefly inhibited by these stimuli' (Mason, 1997). It could additionally be the case in this and associated studies that the immuno-detection techniques used were not sensitive enough to detect low expression levels of 5HT. A linear discriminant function employing background discharge characteristics as independent variables was used to identify serotonergic neurones and non-serotonergic neurones (with a 13 out of 13, and 32 out of 33 success rate respectively) with an estimated probability of miscalculation cited as less than 10% (Mason, 1997). However, many functional groups represent much less that 10% of a total population (this is the case for example with primary sensory neurones), and so depending on interpretation, this level of accuracy could be seen as leaving significant room for error and thus misclassification.

A third potential caveat is the anatomical location of recordings made in different studies; in experiments where it was concluded that neither On or Off cells are serotonergic, *in vivo* recordings were made at the level of the facial nucleus (Potrebic et al., 1994), whilst in experiments with different conclusions (Pan, 1993),

RVM slices were taken from the rostro-caudal level of the superior olivary complex. Thus it is possible that distinct classes of RVM neurones differ in their neurochemical signature, and in particular their 5HT content, along the rostro-caudal axis. In association with this conclusion, *in situ* hybridisation experiments have shown that the location of cells expressing MOR transcripts is relatively caudal within the RVM (Porreca et al., 2001), which is consistent with early autoradiographic confirmation of the location of medullary MOR expressing cells (Bowker et al., 1988, Bowker and Dilts, 1988, Peckys and Landwehrmeyer, 1999). Finally, it is possible that some or all of the neurones classified as neutral cells in studies that concluded that only neutral cells are serotonergic (Potrebic et al., 1994)(Winkler et al., 2006) may appear functionally different in other studies and preparations. It has already been said in Section 1.3.3.3 that neutral cells may somehow 'become' On cell-like during pathology (Urban et al., 1999), and in Section 4.4 that anaesthetic level may influence the responsiveness of RVM neurones, thus cells that were identified as neutral in the Portrebic study could well have different properties and appear more On cell-like in the unanaesthetised preparation. In support of this it has been reported that some On cells in awake animals adopt characteristics of neutral cells following anaesthesia and are therefore unresponsive to somatic stimulation (Oliveras et al., 1991), yet that is not to say that there is no consistency in the neutral cell population, or indeed other populations of RVM cells, before and after anaesthesia.

Dermorphin-Saporin

Regardless of the current consensus of serotonergic neurones in the RVM and whether or not these cells may partly represent On cells, all On cells are directly inhibited by morphine (Gao et al., 1998) and these cells largely account for the postsynaptic targets of μ-opioid receptor agonists in the RVM (Fields et al., 1983a, Fields and Heinricher, 1989, Pan et al., 1990, Heinricher et al., 1994). This property enables these facilitatory neurones to be targeted and lesioned with a cytotoxin, saporin, conjugated to dermorphin, a potent MOR agonist isolated from amphibian skin (Broccardo et al., 1981, Braga et al., 1984). Saporin is a plant ribosome-inactivating protein[5] that irreversibly damages eukaryotic ribosomes. In its intact form saporin is not internalised very efficiently by eukaryotic cells and only shows cytotoxic effects at concentrations of 10^{-8}-10^{-6} M (Gasperi-Campani et al., 1989).

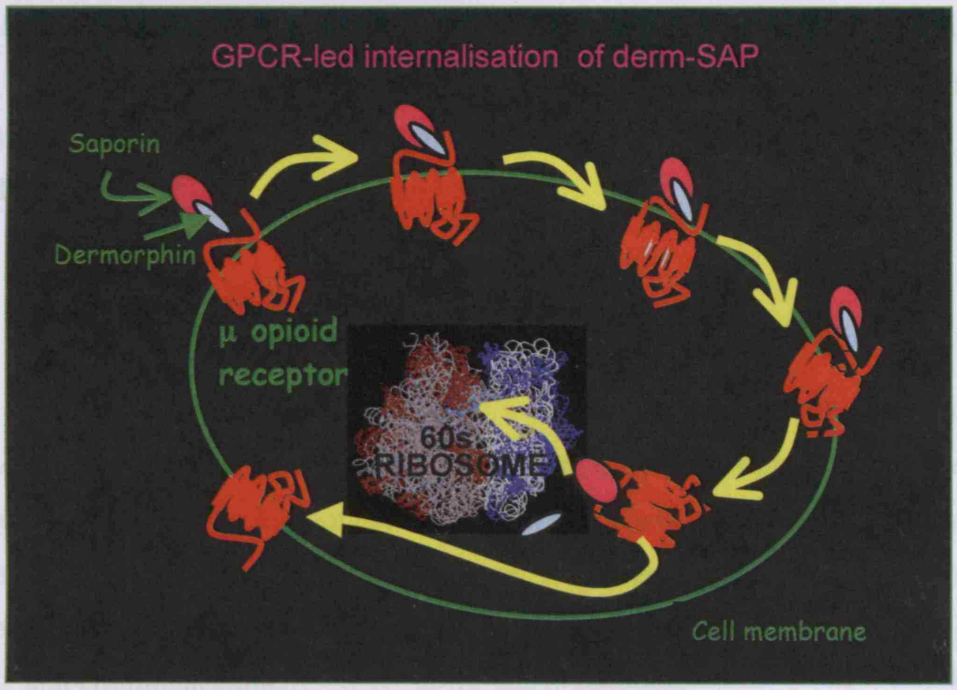
[5] Saporin is taken from the seeds of *Saponaria Officinalis* L. (soapwort).

However, following its conjugation with dermorphin, saporin gains intracellular access to MOR expressing neurones via GPCR internalisation whereupon its toxicity increases several fold, becoming active at concentrations in the range of 10^{-9}-10^{-11}M (Bergamaschi et al., 1988, Cazzola et al., 1991). It exerts its toxicity by interacting with the 60s subunit of ribosomes to inhibit protein synthesis, (Barbieri et al., 1982, Stirpe et al., 1983) which eventually results in cell death via apoptosis (Bergamaschi et al., 1996) (see Figure 6.1).

Saporin has previously been tagged to Substance P to successfully dissect out NK1 receptor-expressing lamina I projection neurones from the spino-bulbo-spinal modulatory circuit (Mantyh et al., 1997, Nichols et al., 1999, Suzuki et al., 2002b). Crucially, tagging the toxin to the ligand does not alter receptor-binding capacity; radioligand binding experiments in membrane preparations transfected with the rat μ-opioid receptor have verified that attaching saporin to dermorphin does not significantly affect the receptor-binding affinity of the latter (Porreca et al., 2001). These studies have also shown that RVM cells expressing MOR transcripts are highly localised in the NRM and the magnocellular reticular nucleus, with lower densities detected in the paragigantocellular reticular nucleus-lateral part and the gigantocellular reticular nucleus. Caudal to the RVM, MOR transcripts are highly localised to cells in the raphé obscurus, the raphé pallidus, the inferior olivary complex, the external nucleus cuneiformis, the medial solitary nucleus, the hypoglossal nucleus, the dorsal motor nucleus of the vagus, the spinal trigeminal tract, and the nucleus ambiguous. 28 days after injection of unconjugated dermorphin, saporin or derm-SAP into the RVM, coronal sections corresponding to the caudal raphé nuclei were processed for MOR mRNA (Porreca et al., 2001). Results showed that the brain slices of rats pre-treated with either dermorphin or saporin alone showed similar densities of labelling for MOR mRNA in the RVM. By contrast, RVM slices from rats pre-treated with derm-SAP showed significantly lower levels of labelled cells, indicating a regional loss of MOR transcripts in these rats. The specificity of the labelling technique for the MOR transcripts was supported by the consistent distribution pattern of labelled cells across multiple brainstem sections in multiple rats. Moreover, brainstem sections from the three groups of pre-injected rats showed similar distributions and densities of labelled cells, which suggests that the loss of labelling correlated with the stereotaxic delivery of derm-SAP into the RVM, and that

this loss was specific to derm-SAP pre-treatment, with tissue necrosis cited as a negligible contributing factor.

Figure 6.1 *A cartoon showing the GPCR internalisation of the dermorphin-saporin (derm-SAP) conjugate. Upon dermorphin binding to the μ-opioid receptor (MOR), the conjugate is endocytosed into the cell. Dermorphin and saporin detach from one another, and whilst dermorphin is recycled to the cell surface, saporin accesses ribosomal machinery and interferes with protein production. As a consequence, the cell dies.*



I used this targeted ablation technique to selectively degenerate MOR-expressing cells in the RVM. My goal was to determine the consequences of this lesion in normal animals, both with respect to behavioural responses, and evoked-responses of deep dorsal horn neurones. In subsequent chapters I will discuss this lesion with respect to neuropathic animals.

6.2 METHODS

6.2.1 DAY 0 - INTRA-RVM DRUG INJECTION

Described in Section 2.2. Briefly, male Sprague-Dawley rats weighing approximately 130g were deeply anaesthetised with ketamine (1.5mg/kg i.p) and were subsequently secured in ear bars in a stereotaxic head-frame such that the top of their exposed cranium was completely level with equal dorso-ventral co-ordinates. A dental drill was used to make a small incision in the skull at bregma –9.0mm following the midline. A Hamilton syringe filled with 1μl of either 3pmol derm-SAP or 3pmol unconjugated saporin was then unilaterally driven slowly down –9.0mm from the top of the cranium, and the drug solution expelled slowly into the brain tissue. A few minutes later the syringe was withdrawn and the skin overlying the skull was sutured back together. Rats recovered in an incubator and were subsequently re-housed in cages.

6.2.2 DAYS 7, 14, 21 & 28 - BEHAVIOURAL TESTING

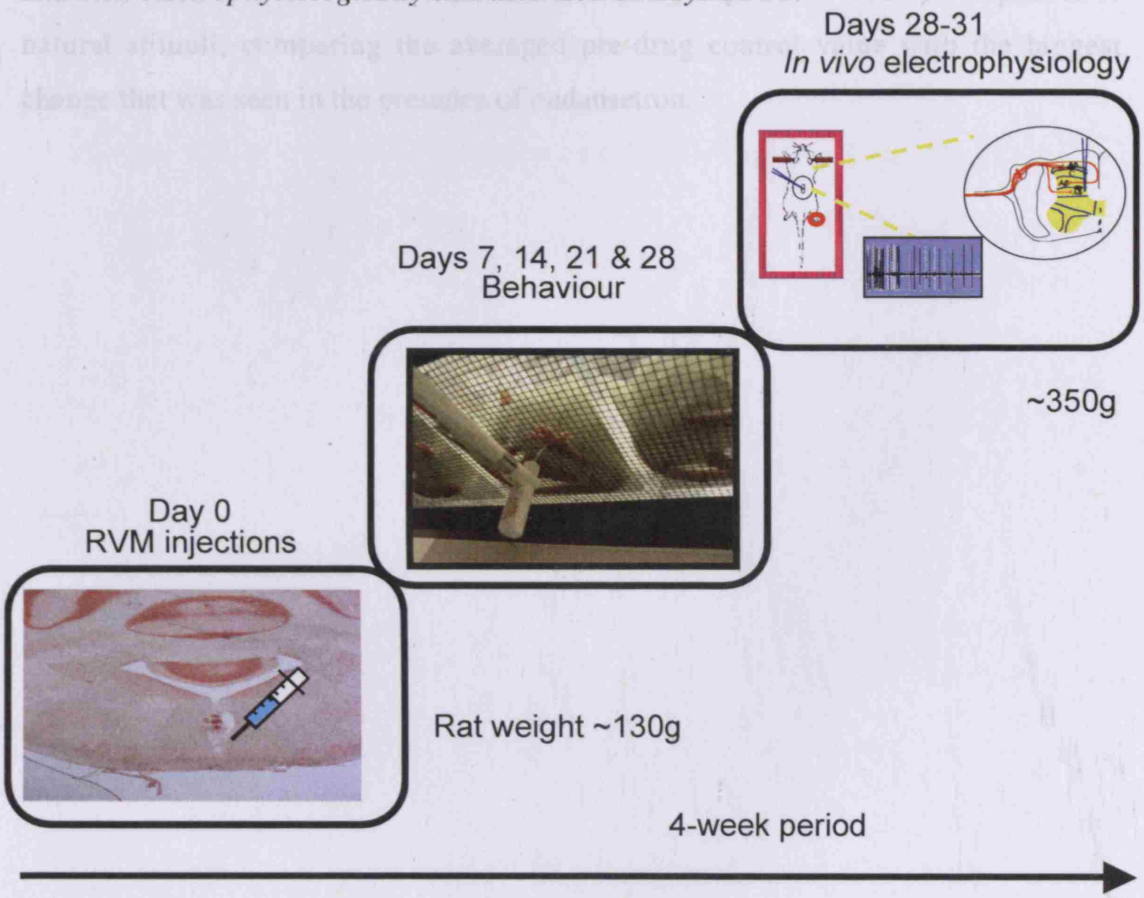
The protocol for behavioural testing was essentially as described in Section 2.4, except instead of testing on various days during the post-SNL weeks, rats used in the present study (which were not neuropathic) were behaviourally tested once a week over the four weeks following RVM injection. Both the left and right hindpaws (as opposed to ipsilateral versus contralateral) were measured in response to vF 1g, 6g, 8g and 15g and cooling acetone.

6.2.3 DAYS 28-31 – ELECTROPHYSIOLOGICAL TESTING & DRUG ADMINISTRATION

In vivo recordings from deep dorsal horn neurones were made on either day 28, 29, 30 or 31 after drug injection. As described in Section 2.5, neurones on either the left- or right-hand side of the central vessel in the spinal cord were characterised in terms of their responses to electrical or natural (mechanical and thermal) stimuli to see whether, and if so how, spinal neuronal responses would differ following the targeted ablation of MOR-expressing RVM cells. Furthermore, in a subset of either

saporin- or derm-SAP-injected animals I administered 50μl of 100μg ondansetron, a $5HT_3$ receptor antagonist, to the exposed surface of the spinal cord following stabilistation of a neurone's responses. Thereafter I re-tested dorsal horn responses to the same peripheral stimuli over a time-course of 80-minutes, carrying out tests at 20-minute intervals to see whether this agent would have the same spinal influence in the absence of RVM MOR cells.

Figure 6.2 *Diagram showing the time-line of procedures used in these experiments. Rats were injected on day 0, behaviourally tested during the following four weeks, and then electrophysiologically characterised on days 28-31.*



6.2.4 IMMUNOHISTOCHEMISTRY AGAINST THE MU-OPIOID RECEPTOR

As described in Section 2.8

6.2.5 DATA ANALYSIS

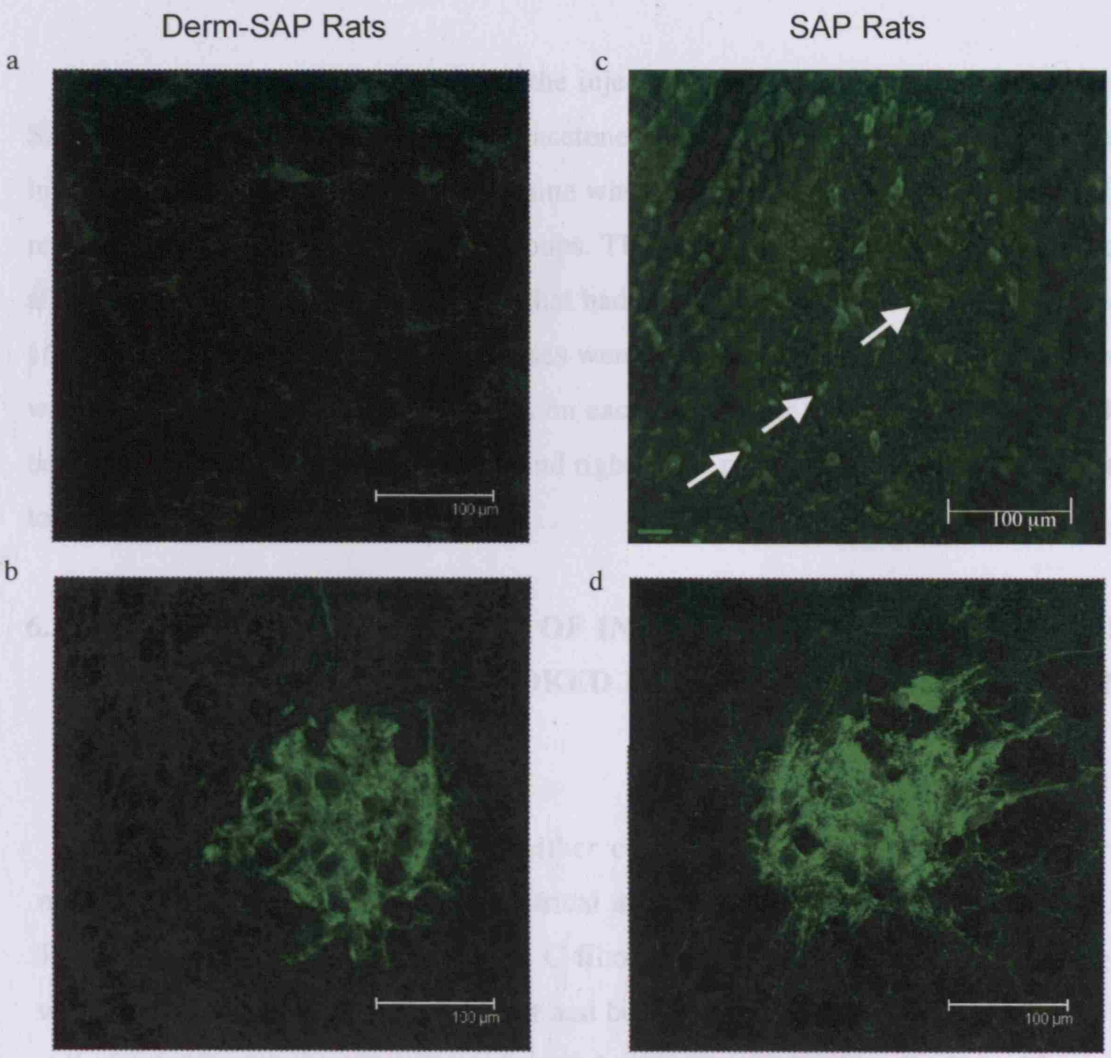
Behavioural characterisation results between the two groups of rats (derm-SAP injected and SAP-injected) were compared using Mann-Witney tests, and data are presented as mean PWD response±SEM. Electrophysiological characterisation data was analysed between groups using unpaired *t*-tests for electrical data, and two-way ANOVA with a Bonferroni post-hoc test for responses to natural stimuli. When looking at whether ondansetron had any effect on neuronal responses within a given group, I used one-way ANOVA and Dunnett's post-hoc tests for electrical responses, and two-way ANOVA with Bonferroni multiple comparisons tests for responses to natural stimuli, comparing the averaged pre-drug control value with the biggest change that was seen in the presence of ondansetron.

6.3 RESULTS

6.3.1 CONFIRMATION OF MOR CELL LOSS FOLLOWING DERM-SAP INJECTIONS

28 days after injection of derm-SAP or SAP into the RVM, immunohistochemistry was carried out against the μ-opioid receptor in brainstem sections corresponding approximately to bregma –9.8. Histological examination of the brainstem sections under a fluorescent confocal microscope confirmed selective loss of MOR-expressing cells in the derm-SAP population relative to the SAP population (compare Figure 6.3a with 6.3c). Successful staining and application of the antibody was confirmed in both groups of rats by a positively stained nucleus ambiguous, an area rich in MOR-expressing cells (Ding et al., 1996) (see figs 6.3b & d).

Figure 6.3 *Intra-RVM injections of derm-SAP resulted in a loss of MOR expressing cells around the NRM.*



28 days after the injection of derm-SAP into the RVM, there was a selective loss of MOR expressing cells in the area corresponding to the NRM (a). The nucleus ambiguous showed a high level of stainging for MOR (b), which indicates that this was not an error of the staining technique. In rats pre-injected with SAP, there was high MOR staining in both the NRM region (c) and the nucleus ambiguous (d). Arrows indicate cell bodies expressing μ-opioid receptors. Scale bar represents 100μm. The images were taken in the Anatomy Confocal Microscopy Suite at UCL.

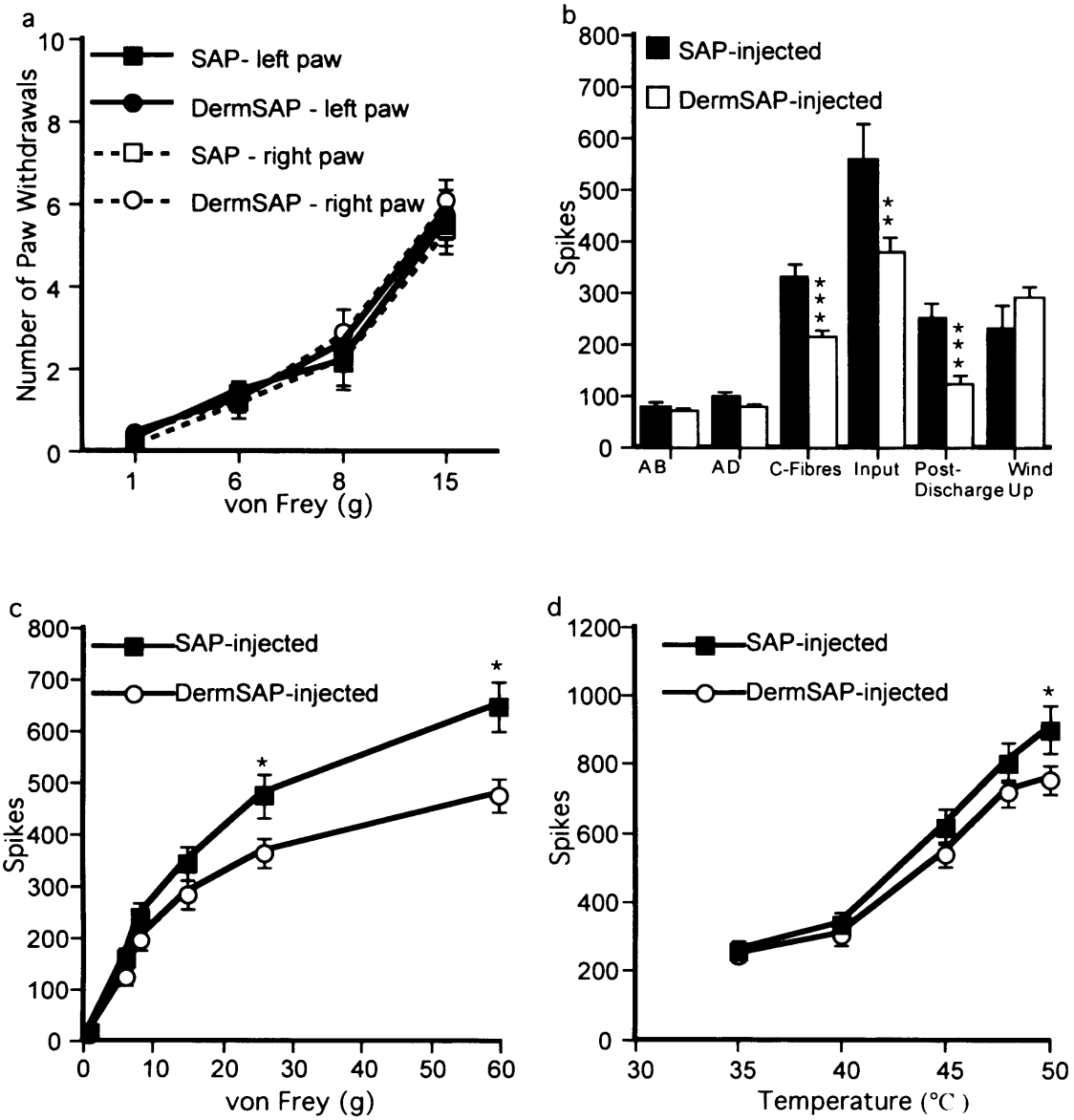
6.3.2 THE RELATIVE EFFECTS OF INTRA-RVM DERM-SAP AND SAP INJECTIONS ON BEHAVIORAL RESPONSES TO MECHANICAL PUNCTATE AND COOLING STIMULI.

On days 7, 14, 21 and 28 after the injection of either 3pmol saporin or derm-SAP into the RVM, PWD responses to acetone, vF 1g, 6g, 8g and 15g were measured in the left and right hindpaws to determine whether there would be any differences in responses between the two injected groups. The data showed that derm-SAP did not affect PWD responses relative to rats that had been injected with saporin (n=16 and 16 respectively) and that PWD responses were consistent for the left and right paw, within and between the testing groups, on each of the four testing days. Figure 6.4a below illustrates this, showing the left and right PWD responses of both groups of rats to a range of von Frey fibres at day 28.

6.3.3 THE RELATIVE EFFECTS OF INTRA-RVM DERM-SAP AND SAP INJECTIONS ON THE EVOKED RESPONSES OF DORSAL HORN NEURONES.

28-31 days after injection of either compound into the RVM, dorsal horn neuronal responses to a range of electrical and natural stimuli were characterised. The results in Figure 6.4b show that C-fibre responses, input and post-discharge were all significantly lower in rats that had been pre-injected with derm-SAP (n=33 cells from 10 rats) than in rats pre-injected with saporin (n=44 cells from 13 rats). Moreover, figs 6.4c & d show that the derm-SAP-injected rats had significantly lower responses to noxious mechanical stimuli (26g and 60g), and 50°C. I therefore conclude that ablating the MOR expressing cells in the RVM with derm-SAP does not affect dorsal horn neuronal responses to threshold stimuli, including brush (not illustrated), yet the lesions do however affect neuronal responses to suprathreshold stimuli. This therefore suggests that On cells control the noxious information that is processed by spinal neurones.

Figure 6.4 *The relative effects of intra-RVM derm-SAP and saporin injections on behavioural PWD responses to mechanical punctate stimuli (a), and dorsal horn neuronal responses to electrical (b), mechanical (c), and thermal stimuli (d) four weeks after injection.*

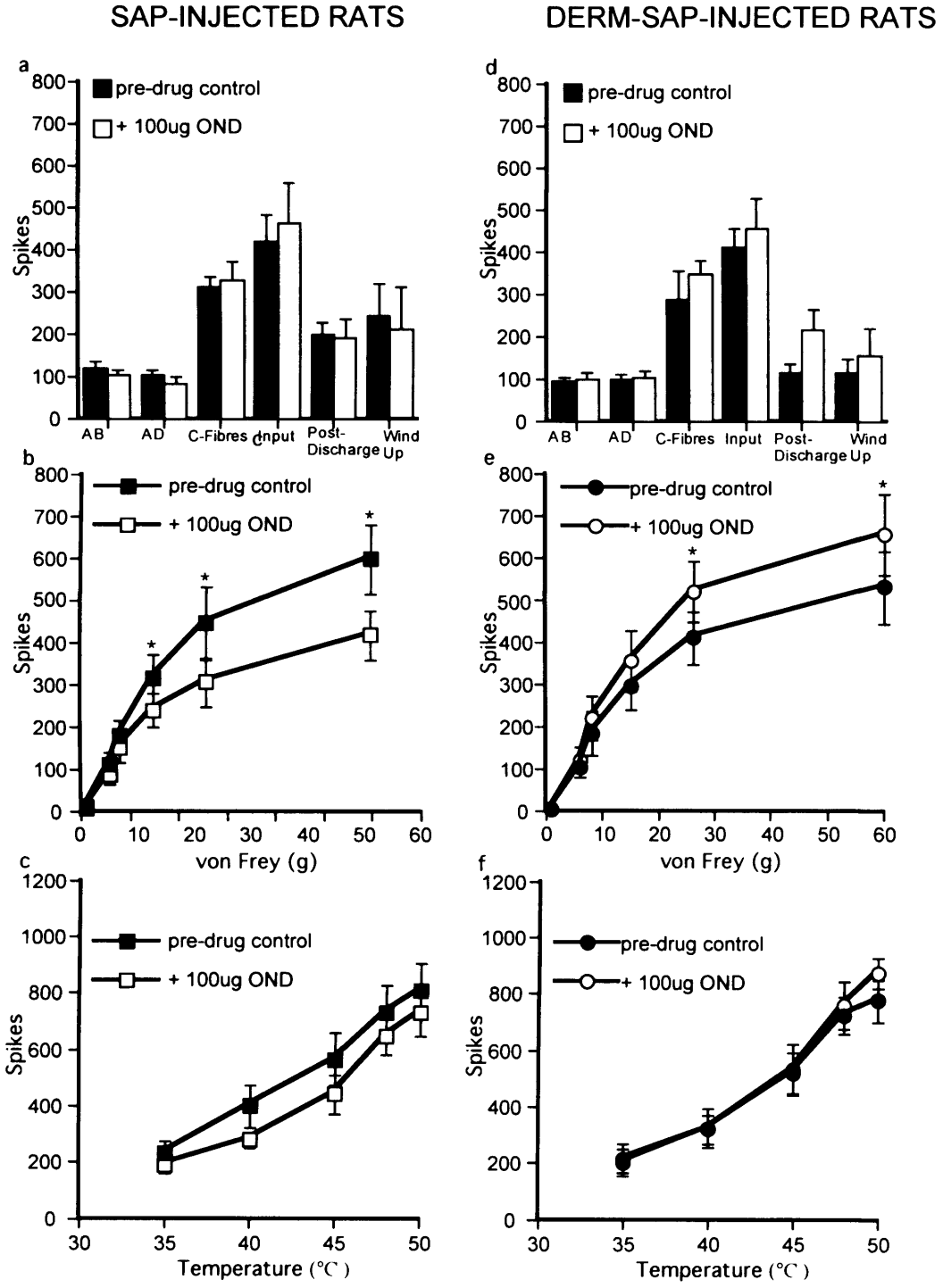


28 days after the injection of 3pmol derm-SAP or saporin into the RVM, PWD responses to mechanical stimuli between dermSAP-injected and SAP-injected rats were not significantly different (n=16 and 16 respectively). This was also true at days 7, 14 and 21 (data not shown) and for responses to cooling stimuli. Four weeks after RVM injections, dorsal horn neuronal responses were characterised *in vivo*. The results show that cells from rats pre-injected with derm-SAP had significantly lower responses to C-fibres, input and post-discharge (b) as well as responses to 26g and 60g (c) and 50°C (d) relative to cells from rats pre-injected with SAP (n=44 and 33 respectively) (*P<0.05, ***P<0.001).

6.3.4 THE EFFECTS OF INTRATHECAL ONDANSETRON ON THE DORSAL HORN RESPONSES OF RATS PRE-INJECTED WITH DERM-SAP OR SAPORIN

Prior to the intrathecal administration of 50μl of 100μg ondansetron, stable control responses to electrical and natural stimuli were established. In SAP-injected animals (n=12), spinal ondansetron did not affect the electrically-evoked responses of spinal neurones (Figure 6.5a). Ondansetron did however significantly reduce responses to vF 15g, 26g and 60g in these same animals (Figure 6.5b), yet did not affect responses to thermal stimuli (Figure 6.5c). Remarkably, in rats pre-injected with derm-SAP (n=13), not only was there no inhibitory effect produced by $5HT_3$ receptor block but spinal ondansetron *enhanced* neuronal responses to vF 26g and 60g (Figure 6.5e). It did not however affect responses to electrical or thermal stimuli (figs 6.5d and 6.5f respectively).

Figure 6.5 *The left-hand panel shows the dorsal horn neuronal responses of SAP-injected animals to electrical (a), mechanical (b) and thermal (c) stimuli both before (filled bar/square) and after (open bar/square) i.t injection of 100μg ondansetron. Likewise the right-hand panel shows the responses of derm-SAP injected animals to the same stimuli before (filled bar/circle) and after (open bar/circle) ondansetron.*



Spinal ondansetron reduced neuronal responses to vF 15g, 26g and 60g in SAP-injected animals, whilst in derm-SAP injected rats it enhanced responses to vF 26g and 60g (*P<0.05).

6.4 DISCUSSION

Before I discuss the results from these targeted ablation studies I will briefly describe the anatomy and physiology of the central serotonergic system and clarify which serotonergic areas may be disrupted by injecting derm-SAP into the RVM.

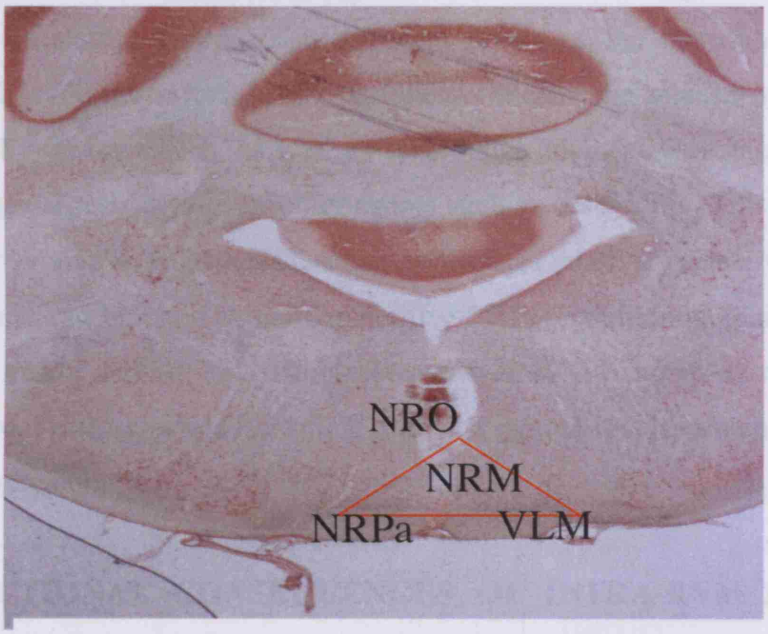
6.4.1 ORGANISATION OF THE SEROTONERGIC SYSTEM

Neurones that produce 5HT make up one of the most complex, phylogenically conserved and widely distributed nervous systems in the brain. The relatively precocious development of this system is reflected by observations that in rats, serotonergic cells are evident by gestational day 12 (Wallace and Lauder, 1983), becoming concentrated by GD 18 into narrow midline sheets within the brainstem (Lidov and Molliver, 1982). Serotonergic cell bodies reside here mainly within the confines of raphé nuclei, which are characterised by a high number of neuronal interconnections, yet there is some displacement of cells into the adjacent reticular formation (Dahlstrom and Fuxe, 1964, Dahlstrom and Fuxe, 1965, Braak, 1970).

By varying degrees of migration, serotonergic raphé cells somehow differentiate into two main neuronal clusters, the superior and inferior groups. The superior group lies within the pons and mesencephalon and contains nuclei that supply the forebrain with 5HT, namely the caudal linear nucleus, the median raphé nucleus and the dorsal raphé nucleus, whilst nuclei that parcel within the inferior group of the medulla - the nucleus raphé magnus (NRM), the nucleus raphé obscurus (NRO), the nucleus raphé pallidus (NRP) and the ventrolateral medulla (VLM), project caudally to the spinal cord and account for the serotonergic innervation seen there. My targeted injection area within the RVM (see Figure 6.6) specifically includes the NRM, a group of large cells that overlaps with the trapezoid body and the dorsal border of the medial lemniscus. The NRM projects neurones bilaterally down the DLF pathway to the dorsal horn to participate in the modulation of sensory transmission (N.B. although the raphé nuclei contain a sizeable proportion of non-serotonergic neurones (Wiklund et al., 1981), nearly all the long projection neurones stem from serotonergic cell bodies). The substantia gelatinosa in particular contains a dense plexus of serotonergic synapses and receptors that receive neurones from the NRM (Ruda et al., 1986, Kwiat and Basbaum, 1992). Furthermore, an anatomical link

between the descending system and WDR neurones in deeper laminae is enabled by the presence of inter-spinal pathways, allowing serotonergic neurones that terminate superficially to innervate more dorsal areas of the spinal cord, (Suzuki et al., 2002b).

Figure 6.6 *A coronal section of the rat brain at bregma –10 showing the locations of the inferior group of 5HT neurones in the brainstem: NRO, NRM, NRP, VLM. The approximate injection area of derm-SAP or saporin used in these studies is mapped out by the red triangle at the end of an injection tract.*



6.4.2 5HT RECEPTORS

In keeping with serotonin's diffuse innervation patterns and diverse functions, there is a large complement of 5HT receptors distributed throughout the neuraxis. To date, 14 unique 5HT receptor subtypes and their related genes have been described in mammals, with many additional species homologues[6]. Different 5HT receptors can mediate reciprocal and even antagonistic actions depending on the membrane and intracellular processes that they are coupled to. Thus some 5HT receptors may stimulate G-proteins that either activate or inhibit cAMP production, whilst others may activate PLC activity. Moreover, the $5HT_3$ receptor, which is prominent within

[6] Species homologues are known as 'orthologous genes'.

the dorsal horn of the spinal cord, gates a cation channel and therefore conducts rapid synaptic depolarisation upon 5HT (or agonist) binding (Gaddum and Picarelli, 1957) (Derkach et al., 1989).

In the RVM, serotonergic projections from the NRM are involved in nociceptive modulation (Taylor and Basbaum, 1995, Bago et al., 2002). The variable capacity of 5HT to inhibit (Yaksh, 1979, Hylden and Wilcox, 1983, Schmauss et al., 1983) or facilitate (Jordan et al., 1979, Hylden and Wilcox, 1983, Vaught and Scott, 1988, Rahman et al., 2004) nociception arises from 5HT acting at multiple (often functionally opposing) receptors at different levels of the nervous system (see (Millan, 2002)). Tissue injury in the periphery induces the release of 5HT which evokes pain by direct means, as well as sensitising nociceptive neurones and thus potentiating the algesic actions of other agents such as bradykinin. This effect can be mimicked in the skin with 2-me5HT, an agent that has relatively selective affinity for $5HT_3$ receptors, and blocked by the competitive $5HT_3$ receptor antagonist tropisetron (Richardson et al., 1985). This simple scenario does not however reflect 5HT's nociceptive modulatory actions at spinal and supraspinal levels, which are by many degrees far more complex.

6.4.3 FUNCTIONAL CONSEQUENCES OF INTRA-RVM DERM-SAP INJECTIONS IN NORMAL RATS

The results from this study show that ablating medullary neurones that express the μ-opioid receptor, many of which are descending serotonergic neurones (Marinelli et al., 2002), does not affect behavioural outcomes to threshold stimuli in otherwise normal animals; the PWD responses of rats that were pre-injected with derm-SAP were very similar to the PWD responses of SAP-injected rats. Consistent with these findings, extracellular recordings from deep dorsal horn neurones showed that responses to peripheral mechanical stimuli within the behavioural range (1g-15g) were similarly unaffected by derm-SAP pre-treatment. Moreover, responses to low-threshold Aβ-fibres, as well as Aδ-fibres were reasonably consistent between the two groups of injected rats. This lack of effect (of derm-SAP) may be due to the preferential expression of $5HT_3$ receptors on the nerve terminals of small-diameter primary afferent fibres, as opposed to the large-diameter 'touch' fibres (Glaum and

Anderson, 1988, Kidd et al., 1993, Tecott et al., 1993, Zeitz et al., 2002), which suggests that 5HT descending from the brainstem does not ordinarily affect low-threshold mechanical input. In contrast, responses of 2nd order neurones to electrical stimulation of C-fibres were significantly reduced as a consequence of derm-SAP injection and medullary MOR cell ablation, as were input, post-discharge and responses to noxious mechanical (26g and 60g) and thermal (50°C) stimuli. This differential effect infers a clear selectivity in the modulation of noxious versus innocuous stimuli from the raphé nuclei, suggesting that low-threshold inputs are subject to less supraspinal control than higher-threshold input. The unaffected wind-up response in the derm-SAP rats re-confirms the lack of descending influence on this spinal measure.

The discriminant actions of descending circuits may also translate to the modality of the peripheral stimulus, since in the present study neuronal responses to noxious 45°C and 48°C were not particularly affected by RVM derm-SAP injections (although responses to 50°C were). In conjunction with this observation, ondansetron, an agent that competitively blocks $5HT_3$ receptor function, has been shown to have more marked inhibitory effects with respect to mechanical evoked-responses than thermal evoked-responses in naïve animals, an effect that is enhanced after nerve injury (Suzuki et al., 2004). Furthermore, inactivating the RVM or transecting the spinal cord significantly reduces mechanical hypersensitivities in nerve-injured animals (Bian et al., 1998, Kauppila et al., 1998, Sung et al., 1998), yet does not alter thermal hyperalgesic responses (Bian et al., 1998). This seems to suggest that like wind-up, local spinal circuits primarily mediate thermal hypersensitivities as opposed to descending circuits. Immunohistochemical data has shown that just 13% of $5HT_3$ receptor-expressing primary afferent fibres co-express the heat-responsive TRPV1 receptor (Zeitz et al., 2002), which partially explains these findings. In addition to this peripheral explanation of why thermal responses are relatively unaffected by manipulations that alter descending circuits, it has also been shown that extracellular concentrations of brainstem monoamines, including 5HT, are not affected by peripheral thermal stimuli (Karlsson et al., 2006). The relevant study looked at the autonomic integration of cardiovascular responses with nociception, and showed that noxious *mechanical* stimulation resulted in increased extracellular 5HT in the RVLM (which probably reflects neuronal release), an effect that was paralleled by increases

in heart rate and mean arterial blood pressure. Thermal stimulation of the periphery elicited similar cardiovascular responses but did not influence the concentration of monoamines in the brainstem. This study confirms the coupling between nociception and pressor responses (Lovick, 1986, Siddall et al., 1994), yet perhaps more saliently it points towards a sub-modality specificity of the brainstem's responses to, and regulation of, noxious stimuli.

So far my results accede previous conclusions from our laboratory that an intact spino-bulbo-spinal circuit, which is driven by NK1 receptor-expressing lamina I neurones, is essential for the full coding of polymodal inputs in the dorsal horn of the spinal cord (Suzuki et al., 2002b). I have extended these observations by showing that this coding depends on activity in the RVM, and most probably On cells. The fact that changes seen in SP-SAP and derm-SAP rats occurred in the absence of any pre-existing injury (such as nerve damage) is further testament to the existence of tonic facilitatory influences from the RVM (Bee and Dickenson, 2007).

It has been shown that 5,7-DHT, a neurotoxin that depletes cells of 5HT without influencing other neural and non-neuronal systems (Duan and Sawynok, 1987), reduces the responses of dorsal horn neurones to peripheral input four weeks after its intrathecal injection in normal animals (Rahman et al., 2006). This study, together with observations that the reduced neuronal responses displayed by SP-SAP rats can be mimicked in SAP-control rats by spinal ondansetron, points to the pharmacological source of the descending facilitations. Indeed, in line with this tonic influence, serotonergic neurones in the brainstem have a rhythmic 'bioelectric signature' that is underscored by a slow discharge pattern (~1-2 spikes/s) (Aghajanian et al., 1978), which is reminiscent of the regular discharge pattern that characterises RVM On cells (Fields et al., 1983a). This patterned activity and the constitutive release of 5HT (inferred by basal 5HT levels in spinal dialysate fluid (Sorkin et al., 1988)) supports a modulatory role of the RVM as opposed to a system that transiently militates against opposing influences. As suggested previously, the efferent output of the inferior raphé nuclei has a predominant gain-control function, setting the level of spinal responsiveness according to the autonomic, motor and sensory requirements of the organism (Lovick, 1997). During the 'normal' physiological state, the spinal volume is appropriately tuned by descending influences to detect noxious input. The

increased influence of descending facilitatory controls after nerve injury may reflect compensatory mechanisms in the face of potential sensory loss, in line with a system that attempts to reset a newly-weighted balance (Suzuki et al., 2004, Rahman et al., 2006, Bee and Dickenson, 2007).

Since the $5HT_3$ receptor is a spinal target for the excitatory projections from the brainstem, I subsequently investigated what effect spinal ondansetron would have on dorsal horn neuronal responses in rats previously injected with derm-SAP. If the ablated neurones in the RVM were indeed serotonergic then their removal should alter the actions of $5HT_3$ receptor block since the brainstem manipulation would have removed the endogenous drive to this spinal receptor. In the SAP control animals, 100μg ondansetron predictably reduced neuronal responses to mechanical stimuli as previously described in naïve rats (Suzuki et al., 2004), yet remarkably in derm-SAP-injected rats this dose of ondansetron *enhanced* neuronal responses to vF 26g and 60g.

The biphasic modulatory effects of spinal 5HT on sensory transmission are well described, with some reports extending to observations of a spinal $5HT_3$ receptor-mediated *anti*nociception (Glaum and Anderson, 1988, Alhaider et al., 1991, Crisp et al., 1991). Given that $5HT_3$ gates a cation channel and the reported inhibitory responses could be blocked by either a $5HT_3$ receptor antagonist (zacopride), or GABA receptor antagonists (for example bicuculline), the underlying antinociceptive mechanism likely involves the 5HT-mediated release of GABA from inhibitory interneurones. The number of $5HT_3$ receptors in the dorsal horn of the spinal cord can be greatly reduced by neonatal capsaicin treatment (Hamon et al., 1989) or dorsal rhizotomy (LaPorte, 1991), which suggests that many are located on the terminals of primary afferent neurones, yet those remaining may reflect $5HT_3$ receptors on intrinsic interneurones within the CNS (Kilpatrick et al., 1987). However, given the predominantly pronociceptive effect of 5HT acting at $5HT_3$ receptors, and the clinically efficacious analgesia exerted by tropisetron and ondansetron (Zofran™) (Farber et al., 2000, McCleane et al., 2003), these GABA-dependent antinociceptive effects may ordinarily be masked by the presiding facilitatory influence. Accordingly, intrathecal 5HT is known to have a dose-dependent dual effect on nociceptive processing, with low doses reducing formalin-induced nocifensive responses, and

higher doses increasing them (Oyama et al., 1996). By injecting derm-SAP into the RVM, my intention was to ablate cells expressing the μ-opioid receptor and therefore interfere with the spino-bulbo-spinal circuit, yet as Section 6.1 explains, not all spinally-projecting serotonergic neurones express the μ-opioid receptor, with an estimated quarter (at least) being unaffected by MOR agonists (Marinelli et al., 2002). My brainstem manipulation would therefore leave a significant intact serotonergic input to the spinal cord, which may effect spinal inhibition (reversed by ondansetron) at this lower concentration. This may additionally account for the lower dorsal horn neuronal responses observed in the derm-SAP rats relative to the SAP-injected rats.

The dose-related dual effect of 5HT is also thought to prevail in pathological situations. Following spinal cord injury for example there is a three-fold increase in 5HT-ir fibres in superficial laminae immediately rostral to the lesion site (Bruce et al., 2002, Inman and Steward, 2003) within spinal segments that contribute to allodynic dermatomes (Oatway et al., 2004). Hypersensitive behaviours induced by this injury are maintained via $5HT_3$ receptor activity since ondansetron dose-dependently reduced at-level mechanical allodynia whilst the $5HT_3$ receptor agonist m-CPBG increased behavioural manifestations of nociception. However despite this, exogenous 5HT delivered intrathecally to the injured animals transiently reduced mechanical allodynia, which suggests that the increased serotonergic density in the spinal cord may initially reduce nociception via actions at $5HT_1$ or $5HT_2$ receptors. Nevertheless, the more enduring effects of this enhanced input from the RVM is spinal facilitation, since once again, 5,7-DHT significantly reduced behavioural hypersensitivities and prevented the inhibitory actions of spinal ondansetron.

$5HT_3$ receptors exert a pronociceptive function by enhancing the evoked release of SP, CGRP and Neurokinin-A from the central terminals of primary afferent fibres (Saria et al., 1990, Inoue et al., 1997). This explains the similarity in behavioural responses produced by intrathecal injections of SP *or* 5HT, and the fact that both these effects can be reversed by antagonising the NK1 receptor (Fasmer et al., 1983, Fasmer and Post, 1983). Interestingly however, antinociception via $5HT_3$ receptor activation has been reported in acute pain models (Glaum et al., 1988) (Paul et al., 2001), whilst intrathecal $5HT_3$ receptor antagonists only reduce the second phase of the formalin response (Green et al., 2000, Zeitz et al., 2002). Together these

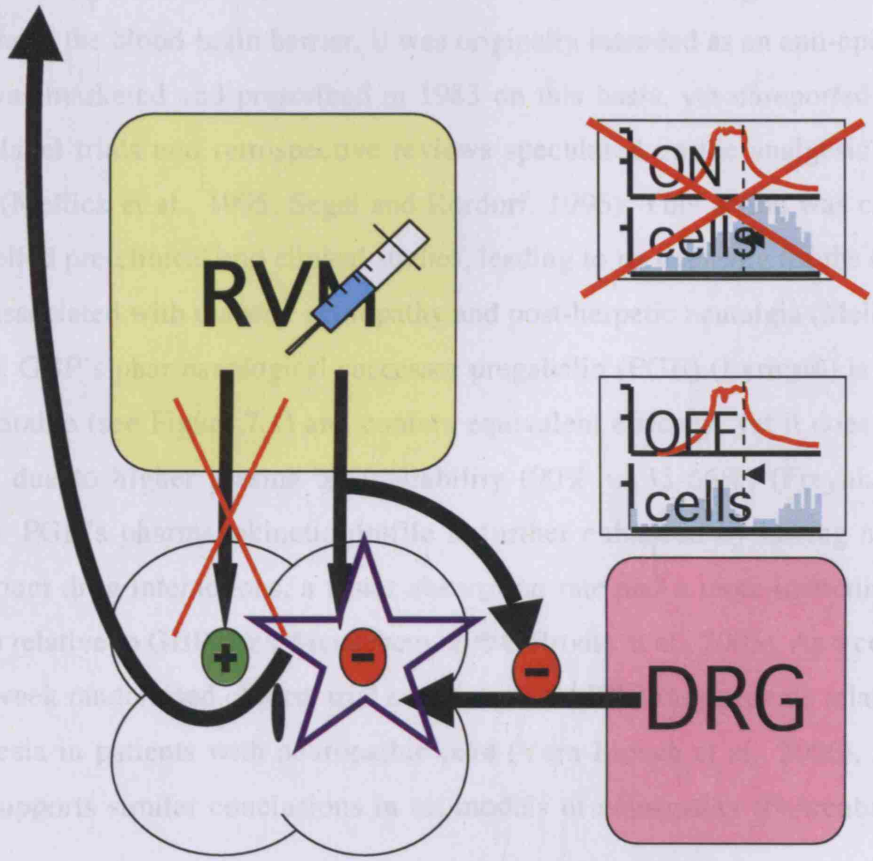
results seem to suggest that $5HT_3$ receptor-mediated spinal facilitation occurs in persistent rather than acute pain states, implying that the supraspinal serotonergic system has a preferential role in maintaining pain states.

In 5HT depletion studies, behavioural hypersensitivities induced by SNL were not reduced (compared to control SNL animals) until post-operative day 7 (Rahman et al., 2006). This correlates with observations from earlier studies that showed that intra-RVM lignocaine, or targeted ablation of RVM MOR expressing cells only affected SNL-induced behavioural hypersensitivities after post-operative day 6 (Burgess, 2002). The mechanisms involved in the medullary MOR cell mediated facilitation include enhanced evoked excitatory transmitter release from primary afferent fibres (Gardell et al., 2003), which is reminiscent of the consequences of $5HT_3$ receptor activation (Saria et al., 1990, Inoue et al., 1997). The alliance between these separate studies, which demonstrate that 5HT depletion or MOR cell ablation manifest pathologically in the same time-dependent way, provides further credence to my results that show an altered serotonergic system following derm-SAP injection in normal animals. The consequences of this injection do not manifest behaviourally but can be seen at the single cell level in responses to suprathreshold stimuli. The fact that dorsal horn responses to C-fibres, input, post-discharge, vF 26g, 60g and 50°C were all significantly lower in derm-SAP injected rats relative to SAP-injected rats highlights the tonic involvement of the descending facilitatory system in setting neuronal responsiveness to high-threshold stimuli, an action which I propose involves the $5HT_3$ receptor. It is entirely plausible that $5HT_3$ receptor block can paradoxically enhance spinal neuronal responses, yet I believe this action is probably masked in the normal setting (and potentially unmasked during the early stages of pain) by a greater descending facilitatory drive. Indeed, lesioning a proportion of the descending serotonergic population revealed this inhibitory role, evidenced by the fact that ondansetron contrarily enhanced evoked neuronal responses in derm-SAP-injected rats and inhibited them in SAP-injected rats.

Given the intricate and mutually affecting balance of supraspinal controls, peripheral input and intrinsic spinal mechanisms that determines the level of neuronal excitability, my next set of studies aimed to look at the consequences of MOR cell ablation in the pathophysiological state, and in particular to extend previous

conclusions from our laboratory regarding the involvement of the spino-bulbo-spinal loop in treatment efficacy (Suzuki et al., 2005).

Figure 6.7 *Summary diagram showing that after injection of derm-SAP into the RVM, dorsal horn neuronal responses to peripheral input are reduced.*



7. THE CONTRIBUTION OF MOR EXPRESSING CELLS IN THE RVM TO THE STATE-DEPENDENT INHIBITORY ACTIONS OF PREGABLAIN

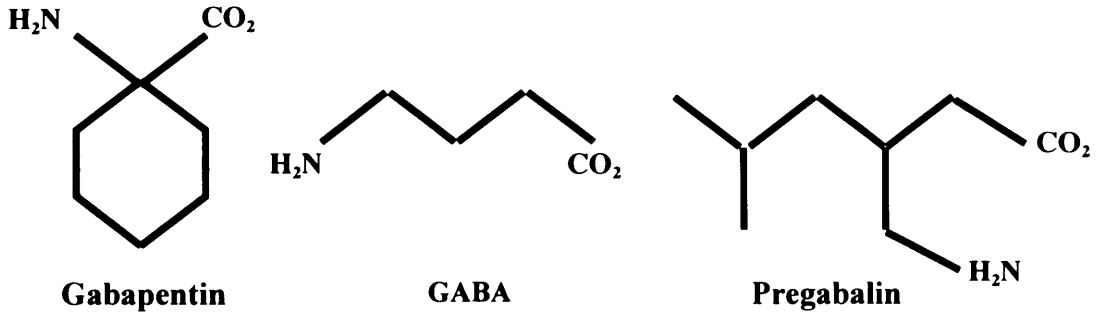
7.1 MECHANISMS OF ACTION OF GABAPENTIN AND PREGABALIN

Gabapentin (GBP) (Neurontin®) is a synthetic analogue of GABA which penetrates the blood-brain barrier. It was originally intended as an anti-epileptic agent and was marketed and prescribed in 1983 on this basis, yet unreported case notes, open label trials and retrospective reviews speculated on the analgesic efficacy of GBP (Mellick et al., 1995, Segal and Rordorf, 1996). This action was confirmed in controlled pre-clinical and clinical studies, leading to its licensing for the treatment of pain associated with diabetic neuropathy and post-herpetic neuralgia (Mellegers et al., 2001). GBP's pharmacological successor pregabalin (PGB) (Lyrica®) is structurally comparable (see Figure 7.1) and confers equivalent efficacy, yet it does so at lower doses due to higher plasma bioavailability (90% v. 33-66%) (Freynhagen et al., 2005). PGB's pharmacokinetic profile is further enhanced by having no clinically important drug interactions, a faster absorption rate and a more immediate onset of action relative to GBP (Ben-Menachem, 2004, Brodie et al., 2005). As a consequence, a 12-week randomised clinical trial concluded that PGB may provide relatively better analgesia in patients with neuropathic pain (Vera-Llonch et al., 2006), an outcome that supports similar conclusions in rat models of neuropathy (Fehrenbacher et al., 2003).

PGB was FDA-approved for the treatment of DN and PHN in 2004, whilst more recently (21st June 2007) it has been licensed for the symptomatic relief of pain in patients with fibromyalgia, a pain state that is clearly not neuropathic since there is no neuroanatomical basis for nerve damage. In addition to DN and PHN, experimental data and anecdotal reports have recommended the use of GBP or PGB in certain other neuropathic pain states, and in particular the pain associated with cancer (Caraceni et al., 2004), spinal cord injuries (Levendoglu et al., 2004), phantom limb phenomena (Bone et al., 2002) and possibly HIV-associated neuropathies (Hahn et al., 2004). Although pooled data and meta-analyses suggest that the analgesic

potency of these agents is lower than that for TCAs and opioids (Finnerup et al., 2005), the increased tolerability, reduced toxicity and lack of abuse potential associated with GBP and PGB offset these differences and sanctions their first-line prescription in many pain states (Backonja, 2002).

Figure 7.1 *The chemical structures of GBP, GABA and PGB*



Despite the diverse therapeutic utility of GBP and PGB (in addition to treating epilepsy and pain, these agents have been shown to have anxiolytic potential (Field et al., 2001), and may also reduce spasticities associated with multiple sclerosis (Paisley et al., 2002)), their mechanism of action has not been fully elucidated. At first it was reasonably assumed that GBP's GABA-mimetic properties would allow it to emulate GABA's inhibitory actions in the CNS, but this was contraindicated by GBP's (and PGB's) lack of binding to either $GABA_A$ or $GABA_B$ receptors (Lanneau et al., 2001) (Taylor et al., 1998, Ben-Menachem, 2004). Furthermore, it has been shown that GBP is not metabolically converted to GABA (Pande et al., 2003), nor is it a substrate or inhibitor of GABA transport (Taylor et al., 1998). It was reported in separate studies that GBP could inhibit GABA-transaminase (Goldlust et al., 1995) or stimulate glutamate dehydrogenase activity *in vitro* (Shimoyama et al., 2000), which therefore inferred effects on GABA levels and glutamate availability, yet the high concentrations of GBP required to do this and the slow onset of activity do not correlate with the fast inhibitory effects of GBP observed at lower doses. Eventually a GBP binding site was isolated from porcine brains and was shown to be the $\alpha_2\delta$ subunit of voltage-gated Ca^{2+} channels (VGCC) (Gee et al., 1996). Since this discovery, other agents modelled on GBP, including PGB and the *R*-isomer of 3-isobutyl GABA have been shown to have analgesic potencies in sensitised states that

correlate with their binding affinity and stereospecificity at the $\alpha_2\delta$ subunit (Dissanayake et al., 1997, Field et al., 2000).

$\alpha_2\delta$ auxiliary proteins are encoded by four different genes ($\alpha_2\delta_{1-4}$) that have tissue-specific distributions (Klugbauer et al., 2003). Their availability is thought to be the rate-limiting factor in the assembly of VGCCs, which may therefore explain why their enhanced expression after nerve injury directly correlates with increased insertion of VGCCs at the cell surface of DRG neurones (Brust et al., 1993). GBP and PGB bind with high affinity to the $\alpha_2\delta_1$ and $\alpha_2\delta_2$ isoforms (Marais et al., 2001, Qin et al., 2002), and in particular depend on an arginine residue located at position 217 in the $\alpha_2\delta_1$ amino acid chain for their interaction; nerve-injured mice with a point mutation at this position (R217A)[7] develop typical behavioural signs of neuropathic pain and respond to morphine and TCAs, yet their hypersensitivities are not sensitive to PGB (Field et al., 1999). This is not due to alterations in the level of $\alpha_2\delta$ protein expression in these mice, it is instead due to a reduced binding affinity of PGB to the substituted subunit (Field et al., 2006). Given that the different (native) forms of $\alpha_2\delta$ subunits do not specifically combine with distinct pore-forming Ca^{2+} channel proteins (for example $\alpha_2\delta_{1/2}$ may be present in L-, N- or P/G-type VGCCs (Stefani et al., 2001, Bertrand et al., 2003)) GBP and PGB theoretically have the potential to exert a global influence on functionally diverse VGCCs throughout the CNS, yet importantly these agents only affect VGCCs associated with hyperexcitable neurones, binding uniquely to them to stabilise their activity, restoring a normal physiological state. Hence GBP and PGB are said to posses 'state-dependent' actions, exerting their inhibitory influence in a regionally selective manner (Dooley et al., 2000, Dooley et al., 2002).

The exact mechanisms underlying this inhibition and analgesic influence are not clear, yet GBP and PGB are known to inhibit Ca^{2+} conductance into neurones, thereby transiently limiting neurotransmitter release and synaptic transmission (Stefani et al., 2001, Sutton et al., 2002). Accordingly, slice recordings have shown that GBP inhibits capsaicin-evoked glutamate and SP release from small afferent fibres entering the spinal cord (Fehrenbacher et al., 2003, Coderre et al., 2005), thus reducing excitatory postsynaptic currents mediated by NMDA, AMPA, mGluR and

[7] Arginine substitutes to alanine at position 217, which may affect glycosylation of the $\alpha_2\delta_1$ subunit and therefore GBP/PGB binding affinity.

NK1 receptors (Shimoyama et al., 2000) in addition to presynaptic excitatory Ca^{2+} currents (Dooley et al., 2000, Fink et al., 2000). Furthermore, prior *in vivo* treatment with the inflammatory agent CFA (or alternatively the PKC activator phorbol 12,13-dibutyrate) enhanced the release of SP and CGRP from spinal tissue *in vitro*, an effect that was attenuated by bath application of GBP or PGB (Fehrenbacher et al., 2003). Together with observations that gabapentin can reduce both the ongoing and evoked activity of dorsal horn neurones in SNL and PSL rats (Chapman et al., 1998a, Pan et al., 1999, Suzuki et al., 2005, Suzuki and Dickenson, 2006), which incidentally is compatible with clinical findings that GBP alleviates both ongoing pain and mechanical allodynia in neuropathic patients (Attal et al., 1990), these results point to a clear spinal action of GBP (and thus PGB too).

There is significant basal expression of the $\alpha_2\delta_{1/2}$ subunit in the spinal cord located at both pre- and postsynaptic sites, yet nerve injury is associated with a preferential presynaptic upregulation of these subunits in the ipsilateral dorsal horn, particularly in high-voltage-activated N-type Ca^{2+} channels (Patel et al., 2000, Luo et al., 2001, Li et al., 2004). This upregulation, which has only been studied in injured DRG neurones, correlates temporally with behavioural hypersensitivities in nerve-injured animals, and spatially with the pattern of PGB binding (Luo et al., 2002, Melrose et al., 2007). Intrathecal delivery of antisense oligonucleotides against the $\alpha_2\delta$ subunit reverses neuropathic pain behaviours (Li et al., 2004), whilst conversely, transgenic mice that constitutively over-express the $\alpha_2\delta_1$ subunit in neuronal tissue, including sensory and dorsal horn neurones, have an enhanced pain phenotype. The neuronal hypersensitivities displayed by these mice can be dose-dependently reversed by i.t GBP which supports the contention that elevated $\alpha_2\delta_1$ subunits at the spinal level not only contribute to abnormal nociception, but also underlie the anti-hyperalgesic actions of GBP (Li et al., 2006). However, the inhibitory actions of GBP and PGB may not be solely due to $\alpha_2\delta$ interactions since GBP (like PGB) has immediate efficacy in acute preparations and short-term inflammatory pain models where $\alpha_2\delta$ upregulation does not occur (Stanfa et al., 1997, Maneuf et al., 2001) (Abe et al., 2002).

There are various hypotheses relating to the 'non-$\alpha_2\delta$' inhibitory actions of GBP and PGB. The first of these states that GBP and PGB may reduce Ca^{2+}

conductance by modulating Ca^{2+} channels through metabotropic mechanisms that involve PKA enzymes (Martin et al., 2002). In turn, this is thought to affect Ca^{2+}-induced Ca^{2+} release from intracellular stores, thus altering Ca^{2+} homeostatic mechanisms (in a similar way that binding to the $\alpha_2\delta$ subunit might). Secondly, it has been proposed that the modulation of VGCCs by these agents may also occur through indirect means, for example GBP and PGB may control the functional expression of these channels at neuronal membranes (Kang et al., 2002). Thirdly, GBP (and therefore PGB) may modulate steady-state non-inactivating K^+ conductance, ATP-sensitive K^+ channels and glutamate receptor function (Stefani et al., 2001) (Cheng and Chiou, 2006). These hypotheses collectively consider the spinal actions of GBP and PGB (Hwang and Yaksh, 1997, Shimoyama et al., 1997, Kaneko et al., 2000), yet they are additionally thought to exert supraspinal antinociceptive effects (Takeuchi et al., 2007). Indeed, pharmacological *f*MRI studies, which reliably detect the central effects of analgesic drugs[8] (Wise et al., 2002), have shown that GBP significantly reduces brainstem activity during central sensitisation in humans (Iannetti et al., 2005). This brainstem site of action is supported by reports that systemic administration of GBP reduces descending influences from the reticular formation onto trigeminal sensory neurones (Kondo et al., 1991). Of course, these effects may not be due to direct actions of GBP on the brainstem, they may instead be secondary to spinal or peripheral actions (since a reduced spinal drive results in decreased spino-bulbo-spinal activity (Suzuki et al., 2002b), yet despite clear pre-clinical demonstrations of GBP's spinal actions, an additional (independent or linked) supraspinal mechanism has been agreed by others (Loscher et al., 1991, Petroff et al., 1996, Tanabe et al., 2005).

The mechanisms underlying the proposed supraspinal actions of GBP involve activation of the descending noradrenergic system (Takasu et al., 2006). Specifically, GBP is thought to presynaptically reduce GABAergic transmission in the locus coeruleus following nerve injury, which consequently releases (via disinhibition) descending neurones that terminate on inhibitory α_2-adrenoceptors in the dorsal horn.

[8] The combination of drug administration with fMRI is recommended in European guidelines for neuropathic pain assessment in human patients
Cruccu, G., Anand, P., Attal, N., Garcia-Larrea, L., Haanpaa, M., Jorum, E., Serra, J. and Jensen, T. S., 2004. EFNS guidelines on neuropathic pain assessment. Eur J Neurol. 11, 153-162.

In conjunction with this theory, supraspinal GBP has been shown to increase NA turnover in nerve ligated animals, whilst central NA depletion with 6-OHDA, or systemic or i.t yohimbine delivery (an α_2-adrenoceptor antagonist) all significantly reduce the analgesic actions of GBP (Tanabe et al., 2005). On the contrary, intrathecal delivery of GBP in neuropathic animals does not alter spinal levels of NA and its metabolites, thus it is thought that *spinal* GBP does not elicit and depend on supraspinal NA release, yet may require basal NA activity to mediate its analgesic effects (which could be why i.t clonidine and GBP interact synergistically against neuropathy-induced behavioural hypersensitivities (Cheng and Chiou, 2006)). Whatever its mechanism, systemic GBP is thought to generate analgesic effects mediated by spinal and supraspinal structures that promote spinal NA turnover.

These studies are not however the only references to GBP's dependency on the supraspinal monoamine system, since the injury-dependent interaction of the descending serotonergic system with spinal $5HT_3$ receptors is also important for the analgesic actions of GBP in neuropathic animals (Suzuki et al., 2005). In particular, a central permissive role exerted by excitatory serotonergic influences from the brainstem on GBP's inhibitory efficacy has been demonstrated, with a hypothesised interplay between presynaptic VGCCs and $5HT_3$ receptors in primary afferent fibres. In SP-SAP rats that had lost a significant proportion of their lamina I projection neurones, and thus had a reduced spino-bulbo-spinal drive (relative to SAP-injected or naïve rats), SNL surgery did not give rise to typical behavioural hypersensitivities during the post-operative period, whilst single unit recordings from deep dorsal horn neurones revealed reduced evoked responses (Suzuki et al., 2002b, Suzuki et al., 2005). Moreover, in SP-SAP SNL rats, GBP was without effect on neuronal responses, which is similar to its lack of effect in naïve animals (Stanfa et al., 1997, Chapman et al., 1998b, Suzuki et al., 2002b). Importantly, whilst GBP's inhibitory efficacy was retained in typically 'pain behaving' nerve-injured SAP animals and 'control' neuropathic animals, this action could be blocked by antagonising spinal $5HT_3$ receptors with ondansetron. Thus it seems that interrupting the spino-bulbo-spinal circuit, either by destroying lamina I neurones or blocking 5HT's excitatory effects in the spinal cord, compromises the inhibitory efficacy of GBP in the injured state. Since nerve injury would have produced the expected upregulation of $\alpha_2\delta$ subunits, at least in the injured nerves, this data clearly shows that increased protein is

not essential for the drug's state-dependency. Pharmacologically mimicking this injury-induced facilitatory drive in either SP-SAP SNL rats or naïve rats by acute treatment with the $5HT_3$ receptor agonist 2-me5HT, enabled GBP to inhibit neuronal responses (Suzuki et al., 2005).

Given the presynaptic location of $5HT_3$ receptors and VGCC in small diameter primary afferent fibres, and that changes occur in both $5HT_3$ receptors and N-type VGCCs after nerve injury (Matthews and Dickenson, 2001b, Cizkova et al., 2002, Wang et al., 2002), it is conceivable, if not highly likely that they interact to co-determine GBP's inhibitory efficacy in sensitised states. In particular, the increased descending input to spinal $5HT_3$ receptors after nerve injury (Suzuki et al., 2004) may depolarise the terminals of primary afferent fibres to the extent that the opening of VGCCs is prolonged, possibly giving GBP increased access to the $\alpha_2\delta$ subunit. This is somewhat analogous to the actions of quaternary local anaesthetic agents and class I antidysrhythmic drugs that enter VGSCs more readily when the channel is opened than when it is closed; their use-dependence and preference for an open channel could be paralleled with the state-dependent actions of GBP (and PGB), explaining in particular how their actions at a ubiquitous channel translates to highly selective inhibition during injury.

GBP's requirement for an intact spino-bulbo-spinal loop, and therefore activity at spinal $5HT_3$ receptors following nerve injury, ultimately means that the same brainstem-reaching circuits that influence nociception also influence treatment outcome. Given this, and the suspected link between medullary MOR expressing cells and serotonergic neurones, I decided to investigate what effect targeted ablation of these medullary cells would have on the evoked responses of dorsal horn neurones in SNL rats, in addition to their responses to pregabalin.

7.2 METHODS

7.2.1 DAY 0 - INTRA-RVM DRUG ADMINISTRATION

1μl of 3pmol derm-SAP or saporin was injected into the RVM of anaesthetised rats as described in Section 6.2.1

7.2.2 DAY 14 – SNL SURGERY

As described in Section 2.3

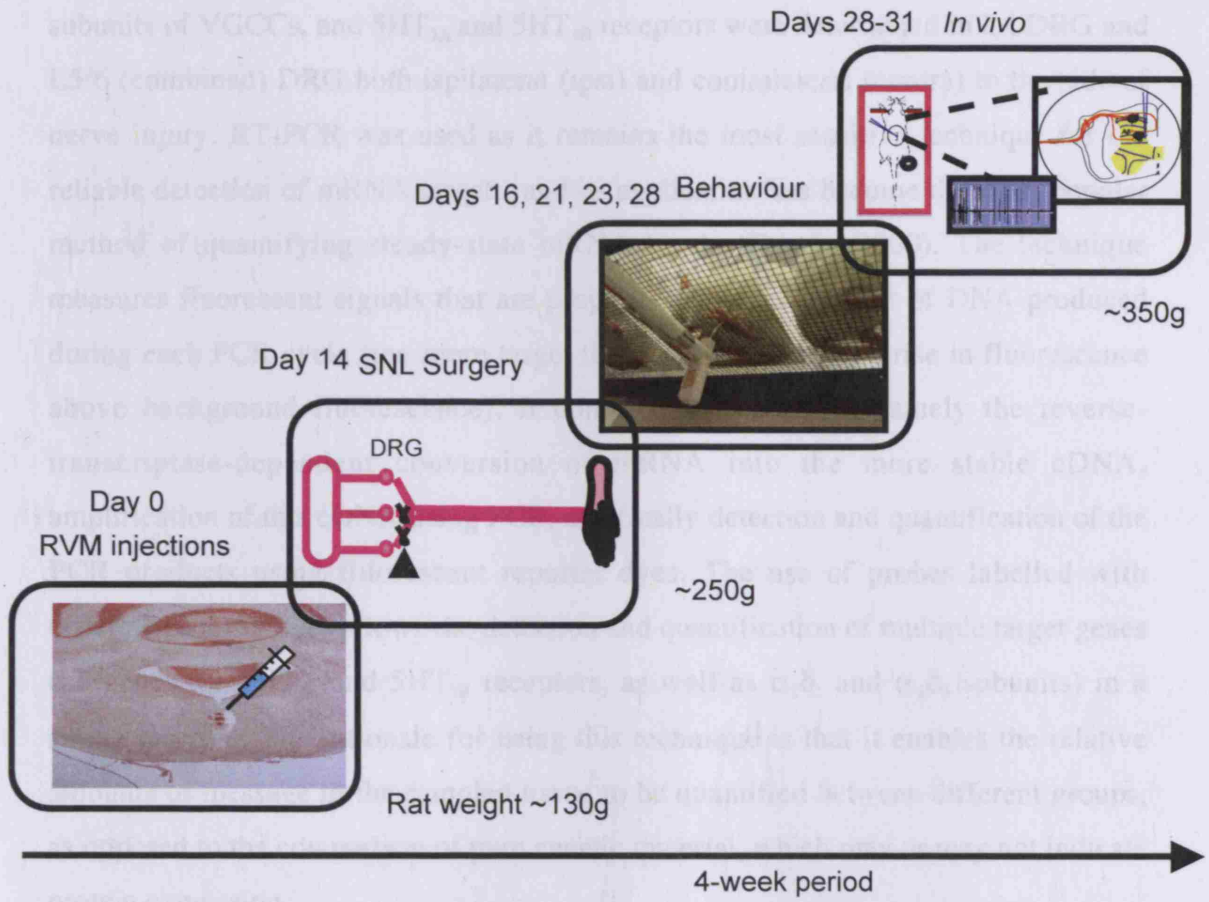
7.2.3 DAYS 16, 21, 23 & 28 – BEHAVIOURAL TESTING

(Days 16, 21, 23 and 28 after RVM injection correspond to days 2, 7, 9 and 14 after SNL surgery). Behavioural testing to assess the development of behavioural hypersensitivities in the ipsilateral hindpaw (relative to the contralateral hindpaw) was carried out as described in Section 2.4.

7.2.4 DAYS 28-31 – ELECTROPHYSIOLOGICAL TESTING AND DRUG ADMINISTRATION

In vivo electrophysiology was carried out as described in Section 2.3, except all neurones recorded from were ipsilateral to the side of nerve injury. Following stabilisation of a neurone's responses, 10mg/kg PGB was injected systemically into the scruff of the rat's neck. Evoked neuronal responses were re-tested every 20 minutes over the course of an hour. Thereafter I applied 50μl of 0.1μg 2-me5HT to the exposed surface of the spinal cord and re-tested responses at t=10, t=30 and t=50 to see whether stimulating spinal $5HT_3$ receptors would influence PGB efficacy in derm-SAP or SAP SNL rats. In a separate group of derm-SAP/SAP SNL rats I applied 50μl of 0.25μg or 1μg morphine sulphate to the exposed spinal cord after neurone stabilisation, and re-tested evoked responses over the course of an hour, to see whether morphine retained its inhibitory activity following medullary MOR cell ablation (for comparison with PGB results).

Figure 7.2 *Diagram showing the time-line of procedures used in these experiments. Rats were injected on day 0, spinal nerve ligated on day 14, behaviourally tested on days 16, 21, 23 & 28, and then electrophysiologically assessed on days 28-31.*



In another set of studies, I deviated from this protocol, and instead of recording dorsal horn activity and responses to PGB two weeks after SNL surgery (i.e. 28 days after intra-RVM injection of SAP or derm-SAP), I looked at neuronal responses just 2 days after SNL surgery (i.e. 16 days after intra-RVM drug injection) to see whether PGB's effect would be any different during the early stages of nerve injury.

7.2.5 REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACRTION (RT-PCR)

RT-QPCR was carried out by Dr. Claudia Bauer (Dept. of Pharmacology, University College London). Under ketamine anaesthesia (1.5mg/kg), DRG tissue

was extracted from normal SNL rats (i.e. rats that had not received injections into their RVM), and derm-SAP SNL rats two weeks after SNL surgery (N.B. these derm-SAP SNL rats had been injected and behaviourally tested in the usual way by myself) and was immediately frozen. Expression levels of mRNA coding for $\alpha_2\delta_1$ and $\alpha_2\delta_2$ subunits of VGCCs, and $5HT_{3A}$ and $5HT_{3B}$ receptors were determined in L4 DRG and L5/6 (combined) DRG both ispilateral (ipsi) and contralateral (contra) to the side of nerve injury. RT-PCR was used as it remains the most sensitive technique for the reliable detection of mRNA targets, and its application has become the most popular method of quantifying steady-state mRNA levels (Bustin, 2000). The technique measures fluorescent signals that are proportional to the amount of DNA produced during each PCR cycle (the more target there is, the greater the rise in fluorescence above background fluorescence). It comprises three steps, namely the reverse-transcriptase-dependent conversion of mRNA into the more stable cDNA, amplification of this cDNA using PCR, and finally detection and quantification of the PCR products using fluorescent reporter dyes. The use of probes labelled with different reporter dyes allows the detection and quantification of multiple target genes (i.e genes for $5HT_{3A}$ and $5HT_{3B}$ receptors, as well as $\alpha_2\delta_1$ and $\alpha_2\delta_2$ subunits) in a single reaction. The rationale for using this technique is that it enables the relative amounts of message in the sampled tissue to be quantified between different groups, as opposed to the comparison of pure genetic material, which may or may not indicate protein expression.

7.2.6 DATA ANALYSIS

Behavioural responses within a group were compared using non-parametric Wilcoxon matched pairs tests. For comparisons between groups (i.e. derm-SAP SNL vs. SAP-SNL), Mann-Witney tests were used.

The effects of drugs on the electrically- evoked responses were calculated using one-way ANOVA followed by Dunnett's post-hoc. The effects of drugs on naturally-evoked responses were tested using two-way ANOVA, and if significant, Bonferroni post-hoc tests.

7.3 RESULTS

7.3.1 MANIFESTATION OF BEHAVIOURAL HYPERSENSITIVITIES IN SNL RATS PRE-INJECTED WITH EITHER DERM-SAP OR SAPORIN

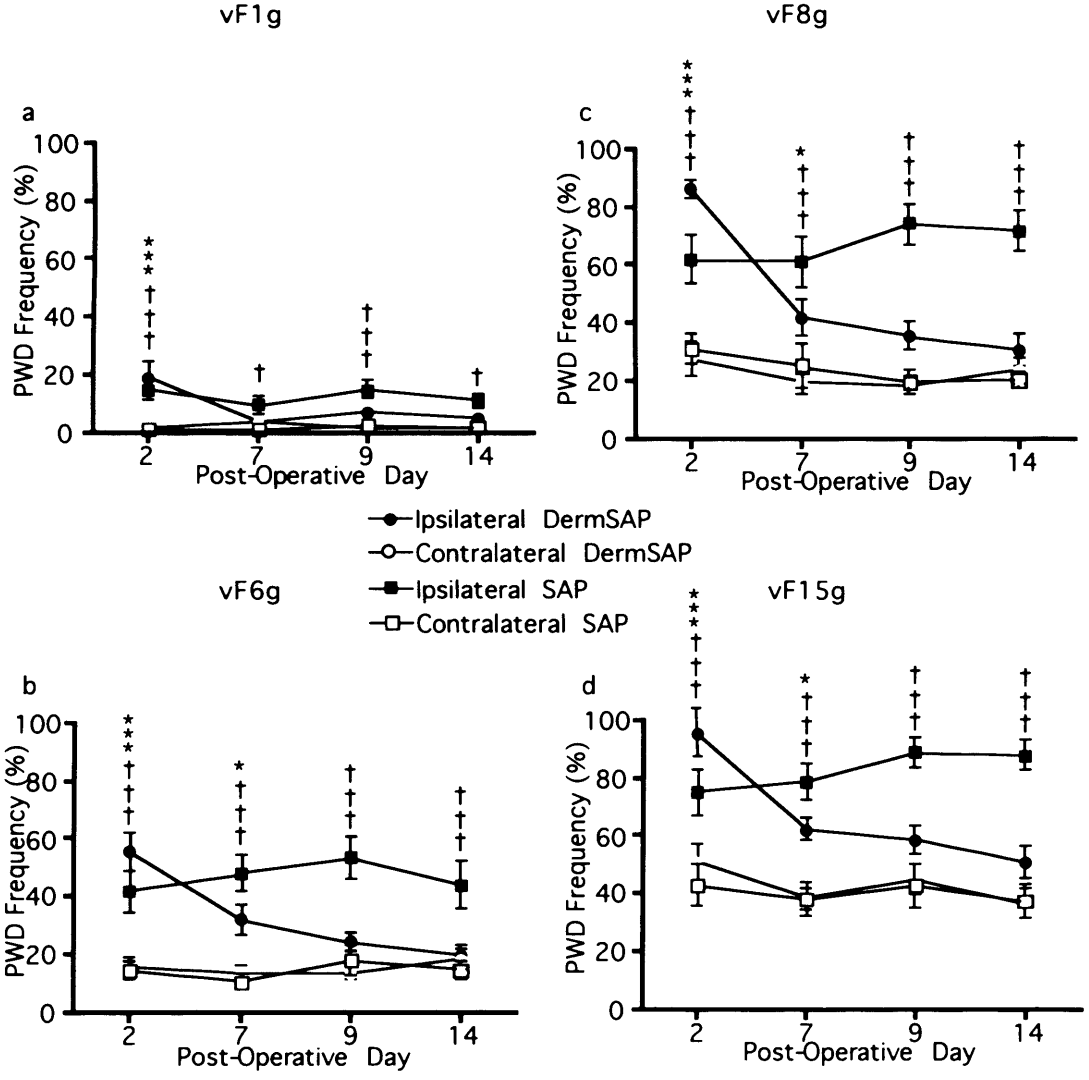
Probing the plantar surface of the hindpaw with a range of von Frey fibres (1g-15g) revealed a rapid development of hypersensitivity in the hindpaw ipsilateral to the side of nerve injury in SNL rats pre-treated (i.e. 14 days prior to nerve ligation surgery) with either unconjugated SAP (n=21), or derm-SAP (n=29) (figs. 7.3a-d). On post-operative (PO) day 2, ipsilateral PWD responses to each of the applied stimuli were reasonably consistent between the two groups of injected rats, and were significantly elevated with respect to their contralateral PWD responses.

PWD frequencies in the ipsilateral hindpaws of SAP SNL rats remained significantly elevated (relative to contralateral paw responses) throughout the 14-day testing period (†P<0.05, †††P<0.001). However, in rats pre-treated with derm-SAP, ipsilateral PWD frequencies steadily returned to 'control' contralateral paw levels after PO day 2 (for example, on PO day 2 the ipsilateral PWD frequency to vF 6g in these rats was 59%, yet by PO day 7 this had declined to 34%, declining still further on PO days 9 and 14 to 25% and 21% respectively). By PO day 7, ipsilateral PWD frequencies in these rats were not significantly different from their contralateral PWD frequencies in response to vF1g (3.3±1.2% and 3.3±1.7%), and by PO day 9, the PWD frequencies of the ipsilateral and contralateral paws of the derm-SAP SNL rats were similar for each of the given stimuli. The ipsilateral PWD frequencies between the two groups of rats were significantly different for all stimuli by PO day 9.

Table 7.1 *The mean PWD frequencies (%) of the ipsilateral and contralateral paws of derm-SAP and SAP SNL rats in response to vF 6g on PO days 2, 7, 9 and 14.*

vF 6g	Ipsi SAP	Cont Sap	Ipsi dermSAP	Cont dermSAP
PO day 2	41.5 ± 7.3	14.2 ± 3.4	58.9 ± 9.5	16.7 ± 5
PO day 7	47.7 ± 6.2	13 ± 3.2	34.4 ± 7.5	14.4 ± 3.8
PO day 9	53.1 ± 7.5	18.5 ± 5.8	23.6 ± 4.1	16.4 ± 3.4
PO day 14	43.8 ± 8.5	14.2 ± 3.6	21.1 ± 4.8	20 ± 5

Figure 7.3 *PWD frequencies of SAP-injected rats (squares, n=21) and derm-SAP injected rats (circles, n=29), comparing ipsilateral responses (filled squares/circles) to contralateral responses (open squares/circles) both within and between groups to vF 1g (a), 6g (b), 8g (c) and 15g (d) on days 2, 7, 9 and 14 after SNL surgery.*
The cross symbol † represents statistically significant differences between the ipsi- and contralateral PWD frequencies of SAP-injected SNL animals.
*The asterisks * represents statistically significant differences between the ipsi- and contralateral PWD frequencies of derm-SAP-injected SNL animals.*

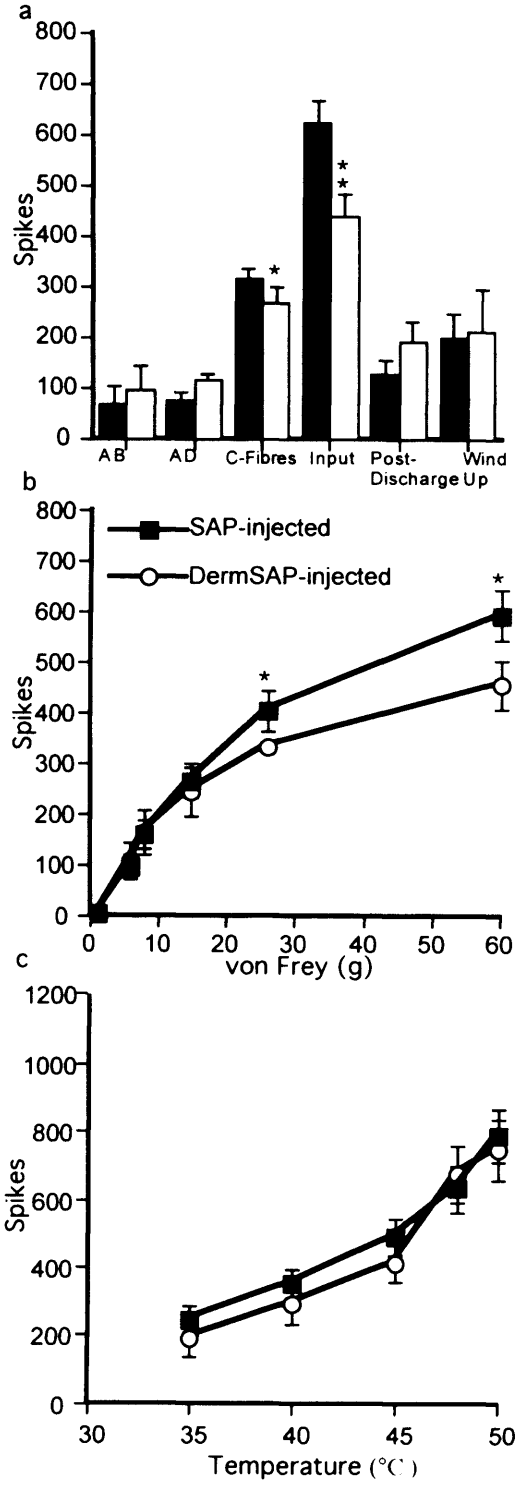


Behavioural hypersensitivities to all stimuli were attenuated in derm-SAP rats by PO day 9. In SAP rats, ipsilateral paw withdrawal frequencies remained significantly elevated throughout the entire post-operative testing period. Differences between ipsilateral paw withdrawal frequencies between the two groups of SNL rats were significant by PO day 9 ($P<0.05$).

7.3.2 DORSAL HORN NEURONAL RESPONSES IN DERM-SAP SNL AND SAP SNL RATS

To investigate whether the response characteristics of deep dorsal horn WDR neurones in nerve-injured animals were altered by ablation of medullary MOR cells, comparisons were made between the baseline responses of neurones from derm-SAP SNL rats (n=16), and SAP SNL rats (n=27) (N.B. recordings were made ipsilateral to the side of nerve injury). The results show that the derm-SAP rats had significantly lower C-fibre and input responses, as well as significantly lower responses to vF 26g and 60g relative to SAP SNL rats. This is similar to the results described in Section 6.3.2 from uninjured derm-SAP and SAP rats, except in Section 6.3.2 I described significantly lower responses to post-discharge and 50°C in the derm-SAP rats, which was not seen here in the presence of nerve injury.

Figure 7.4 *Some evoked neuronal responses accompanying nerve injury are attenuated in derm-SAP SNL rats. The graphs below show dorsal horn responses to electrical (a), mechanical (b) and thermal stimulation of the peripheral receptive field in rats pre-treated with SAP (filled bars/squares) or derm-SAP (open bars/circles).*

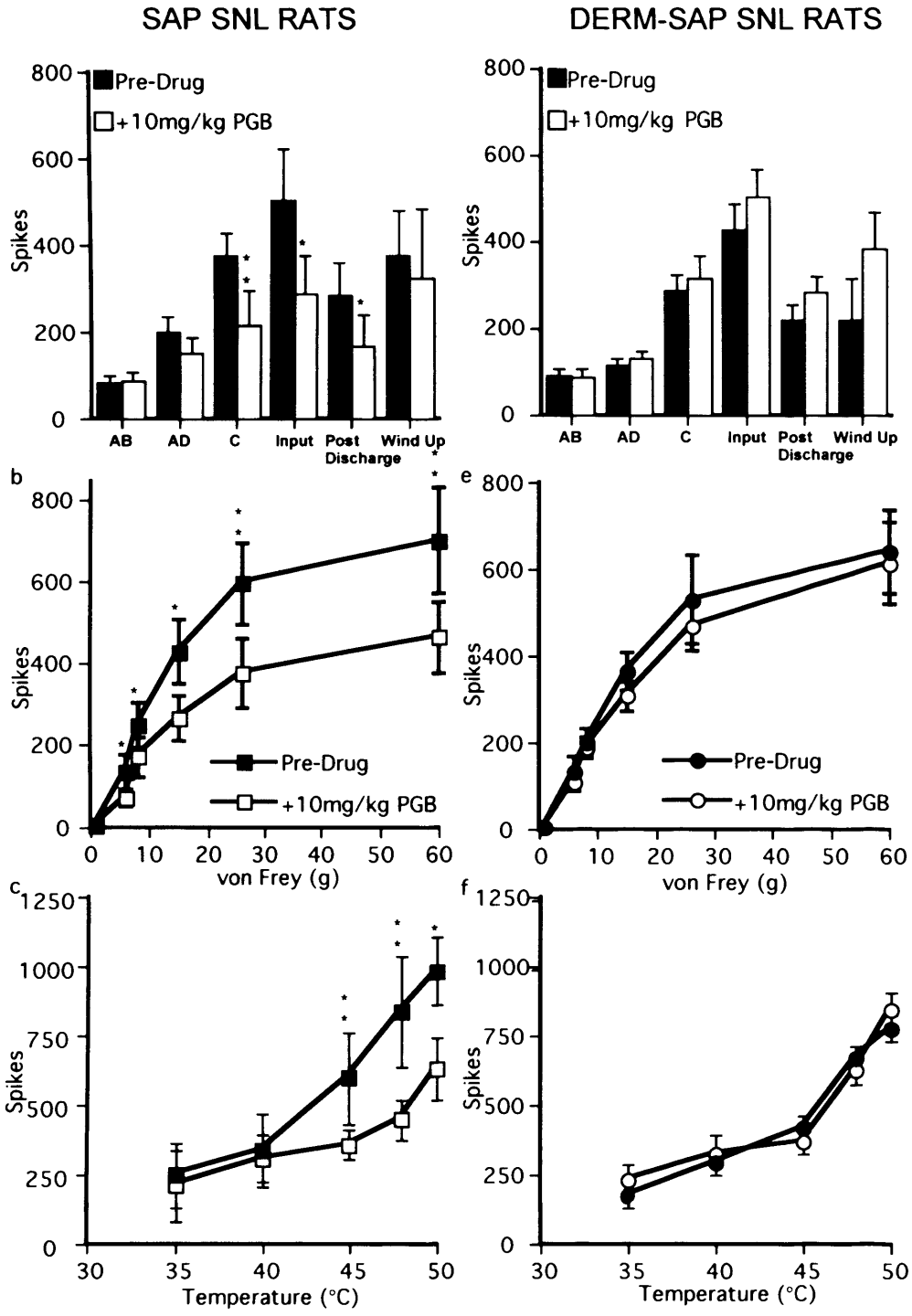


SNL rats pre-injected with derm-SAP had significantly lower C-fibre responses, input and responses to vF26g and 60g relative to SNL rats that had been pre-injected with SAP (*P<0.05 **P<0.01).

7.3.3 PREGABALIN'S EFFICACY IS LOST IN SNL RATS PRE-INJECTED WITH DERM-SAP

In SAP-injected SNL rats, systemic administration of 10mg/kg PGB during electrophysiological testing caused significant reductions in C-fibre responses, input and post-discharge (figure 5a) in addition to dorsal horn neuronal responses to vF 6g, 8g, 15g, 26g and 60g (figure 5b) and 45°C, 48°C and 50°C (figure 5c). This adheres to PGB's state-dependent activity; these rats displayed hypersensitive behaviours that correlate with neuropathic pain, a defining feature of which is an enhanced functional role of pre-synaptic voltage-gated Ca^{2+} channels (Matthews and Dickenson, 2001b). By contrast, in the derm-SAP SNL rats, who had lost hypersensitive behaviours 14 days after nerve injury (i.e. at the time of electrophysiology testing), PGB was without effect on neuronal responses to the complete range of electrical, mechanical and thermal stimuli used (figs. 5d-e). Thus it seems that not only are intact RVM MOR cells critical for maintaining nerve-injury-induced behavioural hypersensitivities but their activity permits PGB's inhibitory actions at the level of the dorsal horn.

Figure 7.5 *Spinal neuronal responses to hindpaw electrical (a,d), mechanical (b,e) and thermal (c,f) stimulation in SAP and derm-SAP rats (left hand panel n = 16 and right hand panel n = 11 respectively) are shown both before (filled bars/squares/circles) and after (open bars/squares/circles) systemic administration of 10mg/kg PGB (* P<0.05, **P<0.01).*

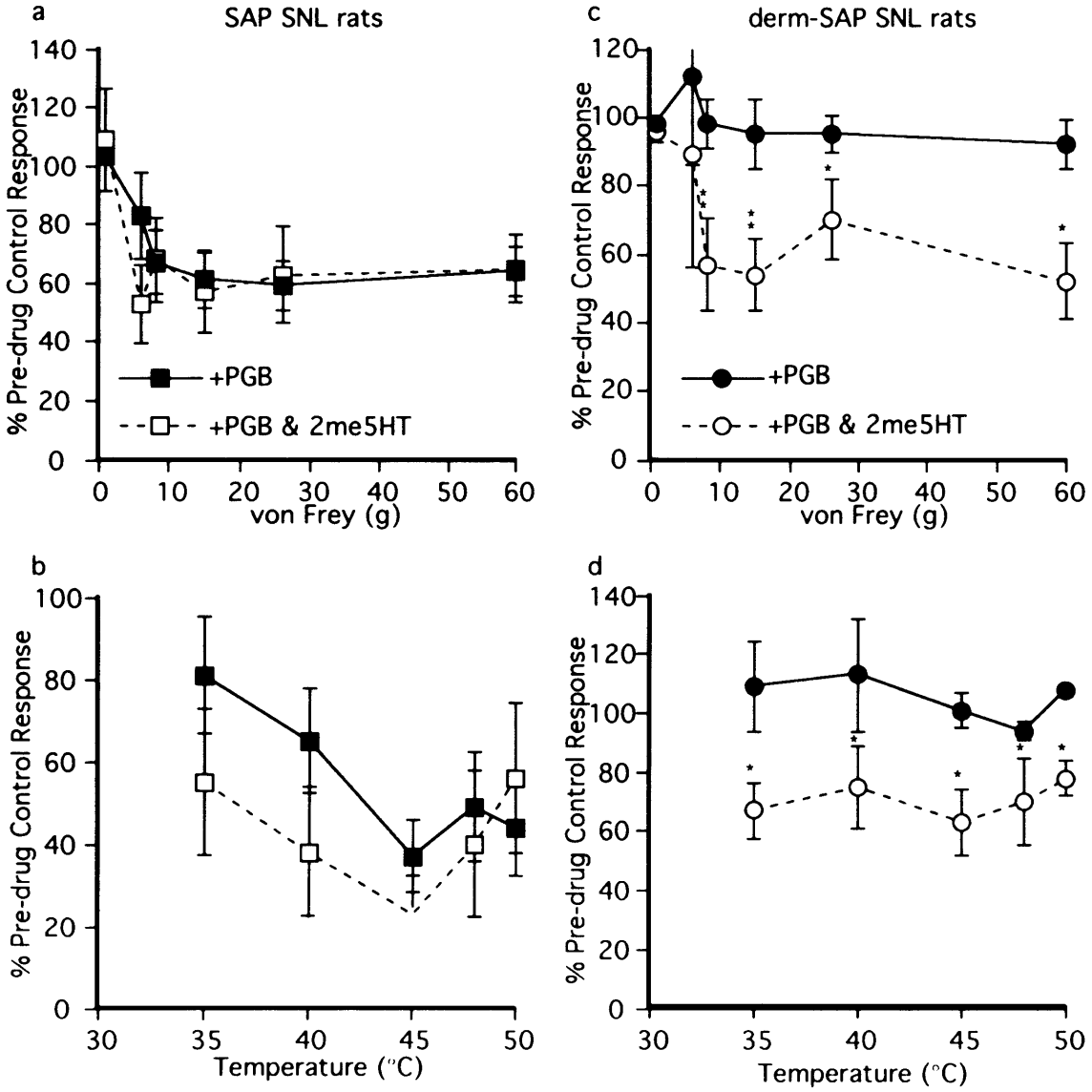


Pregabalin reduced spinal neuronal responses to many of the applied stimuli in SAP SNL rats but was without effect in derm-SAP SNL rats.

7.3.4 RESTORATION OF PREGABALIN'S EFFICACY IN DERM-SAP SNL RATS BY SPINAL $5HT_3$ RECEPTOR STIMULATION

Since a single systemic dose of PGB failed to alter spinal neuronal responses to a range of peripheral stimuli in derm-SAP SNL rats in the way that it did in SAP-injected SNL rats, I applied 0.1μg of the $5HT_3$ receptor agonist 2-me5HT to the exposed spinal cord one hour after the initial PGB injection. This caused a significant reduction in neuronal responses to mechanical and thermal stimuli in derm-SAP SNL rats (figs. 7.6c-d) as well as responses to electrical stimulation (data not shown), comparable to the reductions effected by PGB alone in SAP SNL rats (2-me5HT did not further reduce neuronal responses in these rats, figs. 7.6a-b). Thus in the presence of systemic PGB, the prevention of its inhibitory effects by RVM MOR cell ablation could be overcome by activation of spinal $5HT_3$ receptors. These results uphold similar conclusions from our laboratory that GBP (PGB'S structural progenitor), depends (at least in part) on a descending serotonergic drive from the brainstem for its inhibitory efficacy during nerve injury (Suzuki et al., 2005).

Figure 7.6 *Spinal activation of 5HT$_3$ receptors after systemic administration of PGB reduced dorsal horn neuronal responses in derm-SAP SNL rats. In SNL SAP rats (left hand panel, n = 21), the subsequent addition of 0.1μg of spinal 2-me5HT to PGB (open squares) did not significantly alter neuronal responses with respect to PGB alone (closed squares), whilst in derm-SAP SNL rats (right hand panel, n = 15), 2-me5HT added to PGB induced inhibition of responses to punctate mechanical (c) and thermal (d) stimuli (compare closed circles with open circles) (*P<0.05, **P<0.01). Each data point represents the percentage of pre-drug control response for that stimulus, and the asterisks denote the significance of PGB + 2-me5HT's effects with respect to the effects of PGB alone.*

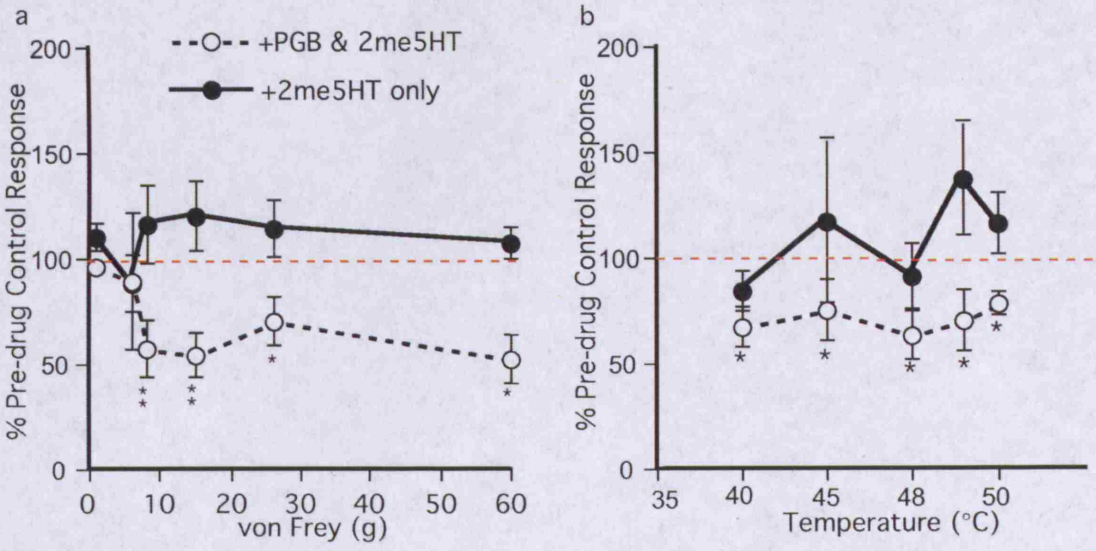


In derm-SAP SNL rats, PGB's lost efficacy was restored by spinal activation of 5HT$_3$ receptors. Spinal application of 2-me5HT did not however alter dorsal horn responses in SAP SNL rats that were already inhibited by systemic PGB.

7.3.5 THE EFFECTS OF SPINAL 2-me5HT ALONE IN DERM-SAP SNL RATS

To verify whether the inhibitions seen after the spinal application of 2-me5HT in derm-SAP SNL rats were the result of permissive actions on PGB's inhibitory effects, or whether they were due to 2-me5HT's effects *per se*, I applied this $5HT_3$ receptor agonist to the exposed spinal cord in a subgroup of derm-SAP SNL rats that had not been injected with PGB one hour previously. The results (Figure 7.7) revealed no direct inhibitory effects of the agonist, thus confirming that spinal 2-me5HT by itself was not responsible for the reduced responses, but that the combination of 2-me5HT with systemic PGB was.

Figure 7.7 *The graphs below show the dorsal horn responses of the derm-SAP SNL rats to mechanical (a) and thermal (b) stimulation in the presence of both PGB and 2-me5HT (open circles and dashed lines), or 2-me5HT alone (closed circles and solid lines). Data are presented as maximum percentage change from the averaged pre-drug control responses.*

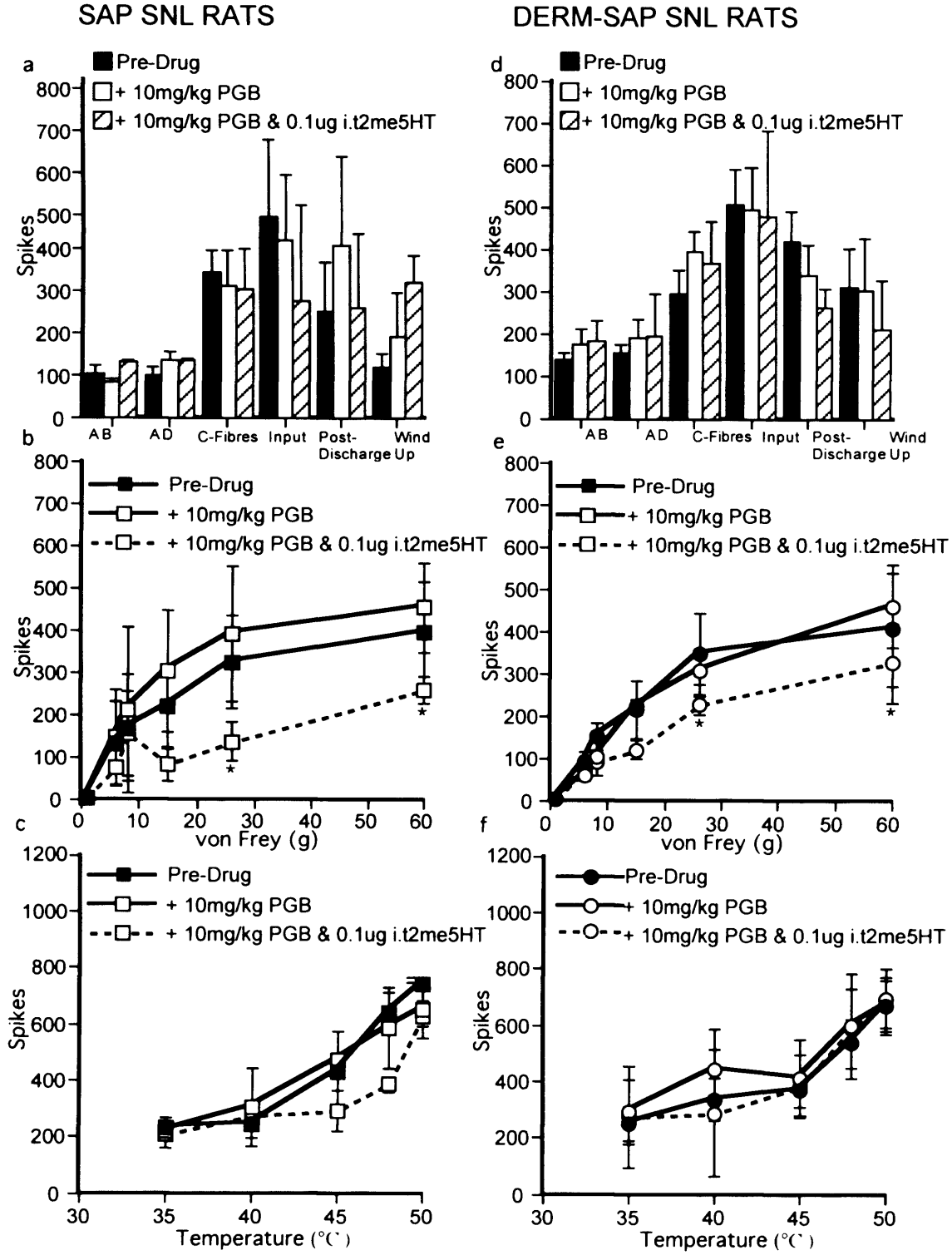


2me-5HT added to the exposed surface of the spinal cord in derm-SAP SNL rats did not significantly alter dorsal horn neuronal responses to any of the applied stimuli (closed circles). However, as seen in figs. 7.6c & d, 2-me5HT added in the presence of systemic PGB did result in significantly reduced neuronal responses (relative to pre-drug control values) (*P<0.05, **P<0.01).

7.3.6 THE EFFECTS OF PGB IN DERM-SAP OR SAP-INJECTED RATS TWO TO FOUR DAYS AFTER SNL SURGERY

To examine the hypothesis that the full expression of neuropathic pain and efficacy of PGB requires descending facilitatory input (and activation of spinal $5HT_3$ receptors), which, according to behavioural studies may only activate several days after nerve injury (see Figure 7.3 above, in addition to (Burgess, 2002)), I looked at the inhibitory capacity of systemic PGB on the evoked responses of dorsal horn neurones two to four days after SNL surgery (i.e. before these descending controls come into play in the maintenance stage) in rats pre-treated with either derm-SAP or SAP, and subsequently the neuronal effects of spinal 2-me5HT in the presence of PGB. The results showed that PGB had no inhibitory effect in either the derm-SAP- or SAP-SNL rats at this early post-operative day (Figure 7.8). However, as at post-operative day 14 in derm-SAP SNL rats, the application of 2-me5HT induced the inhibitory effects of PGB in both groups of animals, hence there were significant reductions in the responses to vF 26g and 60g (figs 7.8b and 7.8e).

Figure 7.8 *Graphs showing the electrical (a, d), mechanical (b, e) and thermal (d, f) evoked dorsal horn neuronal responses of SAP-injected rats (left hand panel, n=9) and derm-SAP rats (right hand panel, n=5) two to four days after SNL surgery, in the presence of PGB, or in the presence of PGB and 2-me5HT.*

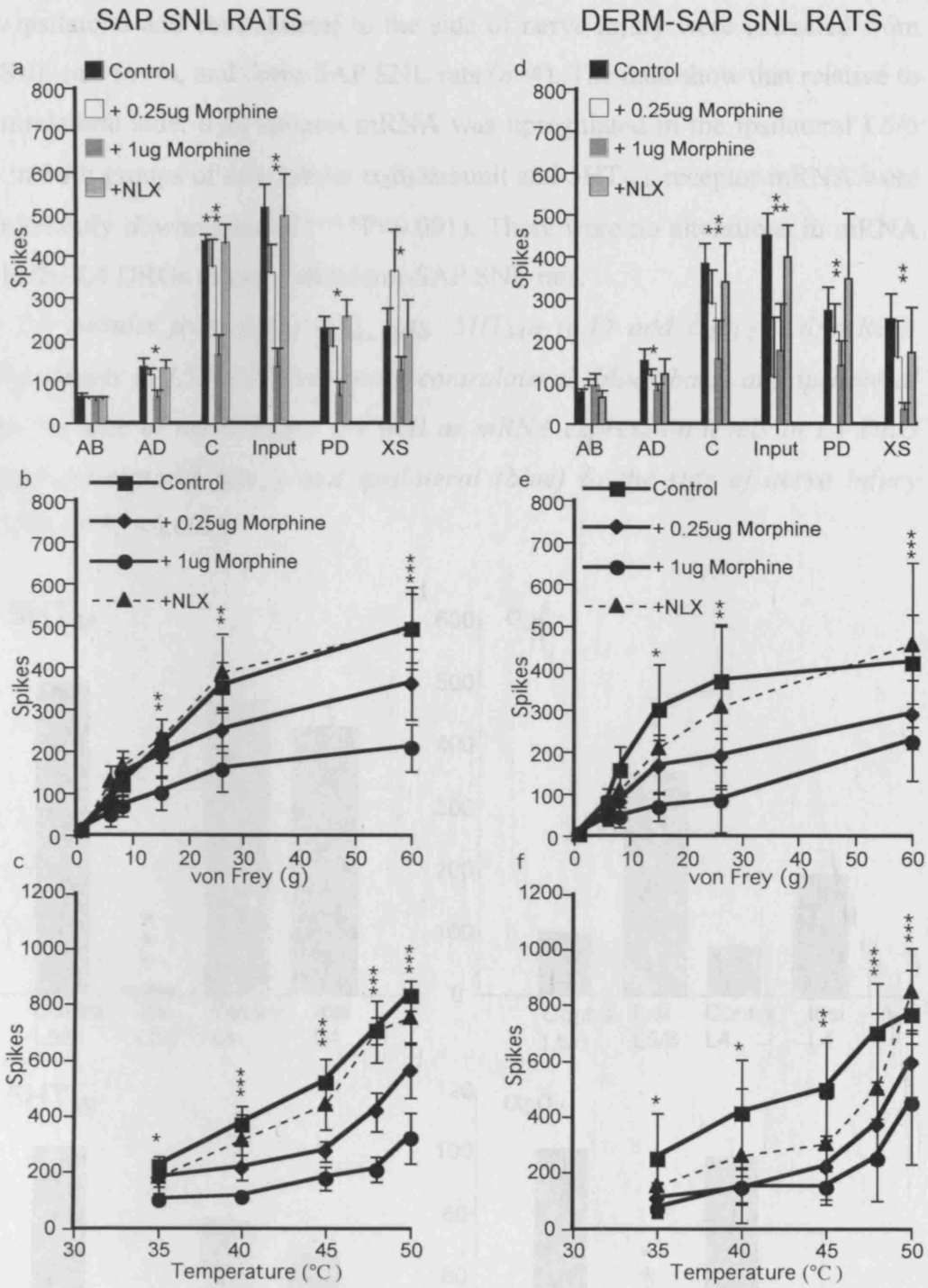


Two to four days after SNL surgery, PGB failed to have any inhibitory effects on the dorsal horn responses of rats pre-injected (14 days prior to SNL) with SAP or derm-SAP. Following the addition of i.t 2-me5HT there was a trend towards reduced neuronal responses in both groups of rats, and these reductions were statistically significant in response to vF26g and 606 (*P<0.05).

7.3.7 THE EFFECTS OF INTRATHECAL MORPHINE IN SNL RATS PRE-TREATED WITH EITHER SAP OR DERM-SAP

To test whether or not the differential effects of PGB seen in SAP and derm-SAP injected rats 14 days after SNL surgery was specific to PGB, or whether it might be a factor of all inhibitory agents, I added morphine to the exposed surface of the spinal cord after neuronal stabilisation to gage the effects on dorsal horn neuronal responses amongst the SAP SNL rats (n =6) and the derm-SAP SNL rats (n = 7). The results in Figure 7.9 show that morphine dose-dependently reduced neuronal responses in both SAP- and derm-SAP SNL rats in a naloxone reversible fashion. However, this inhibiton did not include responses to low-threshold mechanical stimuli (i.e. vF1g, 6g, 8g, 15g), nor did it include Aβ-fibre eveoked responses. At the lower dose of morphine (0.25μg), neuronal responses to natural (mechanical and thermal) stimulation were more significantly reduced in the SAP SNL rats relative to the derm-SAP SNL rats.

Figure 7.9 *Graphs showing the electrical (a, d), mechanical (b, e) and thermal (d, f) evoked dorsal horn neuronal responses of SAP-injected rats (left hand panel, n=6) and derm-SAP rats (right hand panel, n=7) SNL rats in the presence of increasing concentrations of morphine.*



Morphine dose-dependently reduced neuronal responses to many stimuli in *both* SAP- and derm-SAP-SNL rats. Significance stars represent the smallest P value calculated from morphine's inhibitory actions for the given stimulus (*P<0.05, **P<0.01, ***P<0.001). Morphine-induced reductions in neuronal responses were reversed by intrathecal delivery of 50mg naloxone.

7.3.8 QUANTITATIVE PCR AGAINST $5HT_{3A/B}$ RECEPTORS AND THE $\alpha_2\delta_{1/2}$ SUBUNITS IN INJURED AND UNINJURED DRG NEURONES

Two weeks after SNL surgery, DRGs corresponding to the L4 and L5/6 spinal nerves ipsilateral and contralateral to the side of nerve injury were extracted from naïve SNL rats (n=4), and derm-SAP SNL rats (n=4). The data show that relative to the contralateral side, $\alpha_2\delta_1$ subunit mRNA was upregulated in the ipsilateral L5/6 DRGs in both groups of rats, whilst $\alpha_2\delta_2$ subunit and $5HT_{3a\text{-}b}$ receptor mRNA were all significantly *down*regulated (***P<0.001). There were no alterations in mRNA levels in the L4 DRGs of naïve and derm-SAP SNL rats.

Figure 7.9 *Results from naïve SNL rats. $5HT_{3A/B}$ (a,b) and $\alpha_2\delta_{1/2}$ (c,d) mRNA expression levels in L5/6 DRG neurones contralateral (black bars) and ipsilateral (pink) to the side of nerve injury, as well as mRNA expression levels in L4 DRG neurones contralateral (grey) and ipsilateral (blue) to the side of nerve injury (**P<0.01) (***P<0.001).*

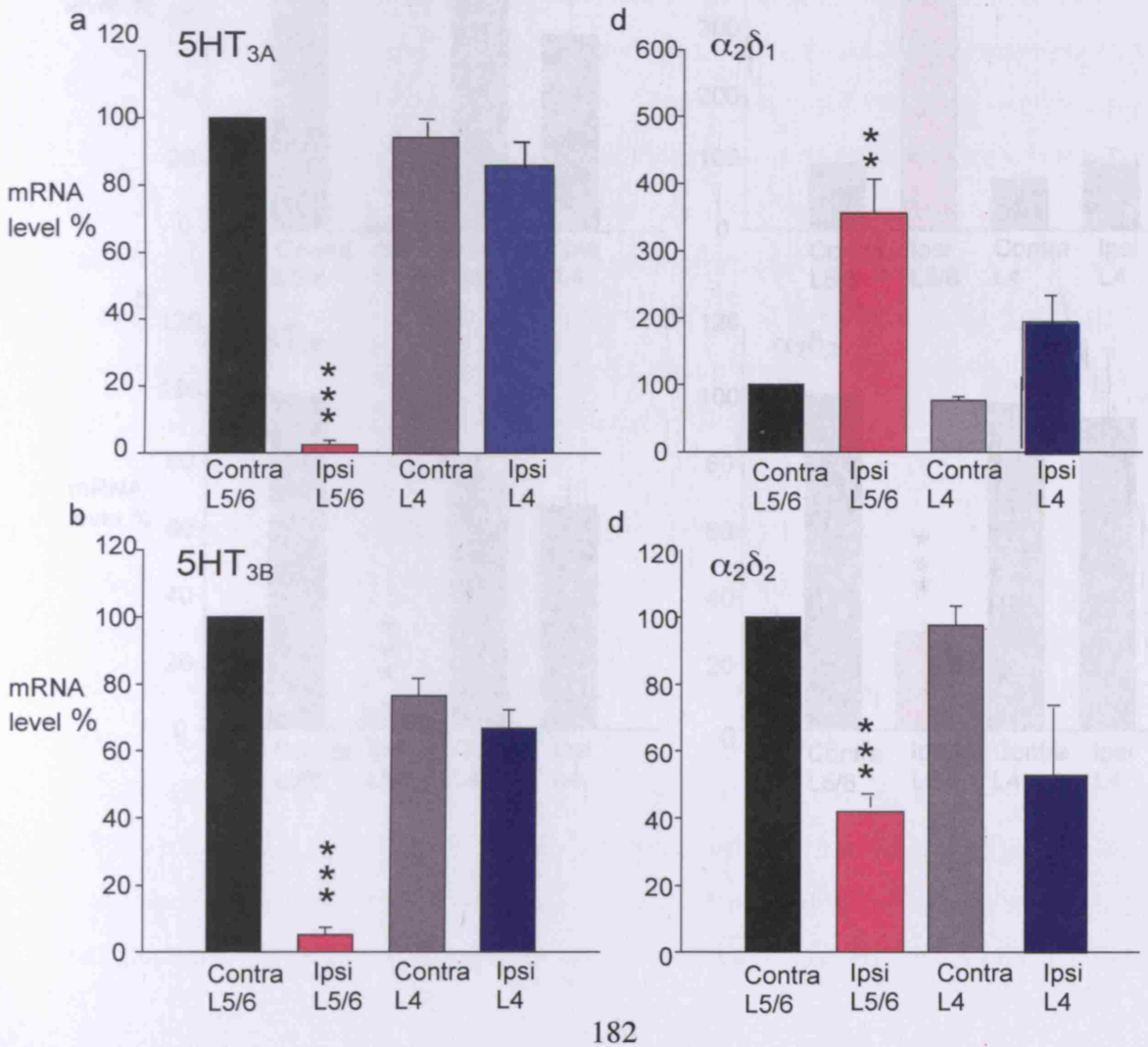
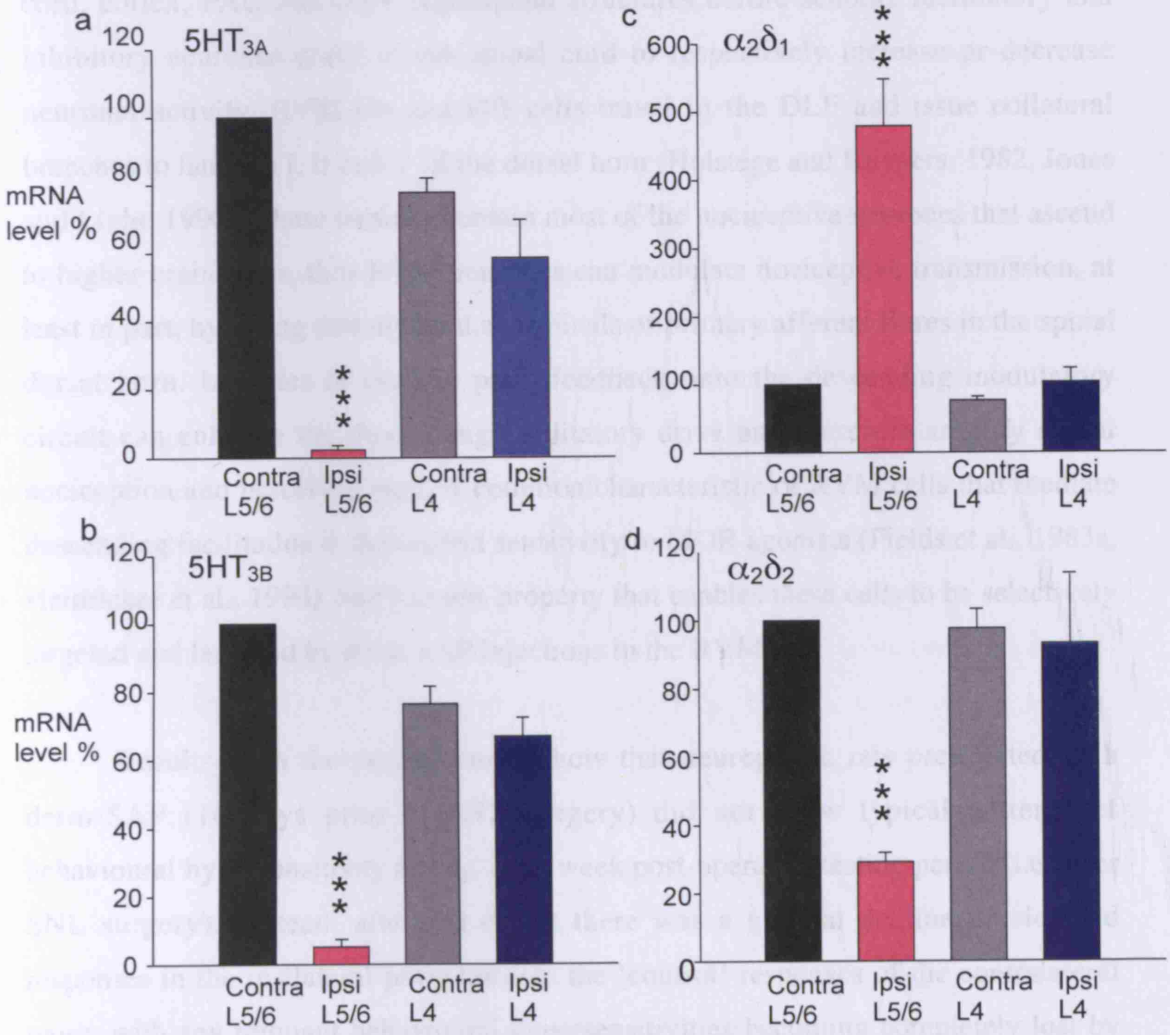


Figure 7.10 *Results from derm-SAP injected SNL rats. $5HT_{3A/B}$ (a,b) and $\alpha_2\delta_{1/2}$ (c,d) mRNA expression levels in L5/6 DRG neurones contralateral (black bars) and ipsilateral (pink) to the side of nerve injury, as well as mRNA expression levels in L4 DRG neurones contralateral (grey) and ipsilateral (blue) to the side of nerve injury. In the ipsilateral L5/6 DRG, $5HT_{3A}$ subunit mRNA was downregulated to just 2.37% of control contralateral levels, whilst $5HT_{3B}$ subunit mRNA was downregulated to 5.1%. The values for the ipsilateral L4 DRG were 75.31% and 87.27% respectively. In the ipsilateral L5/6 DRG, the $\alpha_2\delta_1$ subunit mRNA was upregulated to 478% of control, whilst the $\alpha_2\delta_2$ subunit mRNA was downregulated to 29%. The values for the ipilateral L4 DRG were 125% and 95% respectively. (***P<0.001).*



7.4 DISCUSSION

Central sensitisation, which refers to the increased responsiveness of nociceptive neurones in the CNS to sensory stimulation, has mainly been described as a spinal cord phenomenon that is caused for example by reduced inhibition, excessive primary afferent fibre depolarisation and enhanced strength at excitatory synapses, yet the involvement of supraspinal structures has become increasingly recognised and the results in this chapter show that descending pathways contribute to spinal facilitatory mechanisms and neuropathic pain.

The RVM collects, integrates and filters wide-ranging signals from the spinal cord, cortex, PAG and other supraspinal structures before sending facilitatory and inhibitory neurones down to the spinal cord to respectively increase or decrease neuronal activity. RVM On and Off cells travel in the DLF and issue collateral branches to laminae I, II and V of the dorsal horn (Holstege and Kuypers, 1982, Jones and Light, 1990). These laminae contain most of the nociceptive neurones that ascend to higher brain areas, thus RVM neurones can modulate nociceptive transmission, at least in part, by acting directly on the terminals of primary afferent fibres in the spinal dorsal horn. In states of chronic pain, feedback onto the descending modulatory circuit can enhance the descending facilitatory drive and therefore amplify spinal nociception and perceived pain. A common characteristic of RVM cells that mediate descending facilitation is their direct sensitivity to MOR agonists (Fields et al., 1983a, Heinricher et al., 1994), and it is this property that enables these cells to be selectively targeted and lesioned by derm-SAP injections in the RVM.

Results from the present study show that neuropathic rats pre-treated with derm-SAP (14 days prior to SNL surgery) did not show typical patterns of behavioural hypersensitivity during the 2-week post-operative testing period (i.e. after SNL surgery). Instead, after PO day 2 there was a gradual decline of elevated responses in the ipsilateral paws towards the 'control' responses of the contralateral paws, with any remnant behavioural hypersensitivities becoming completely lost by PO day 9. This is in strong contrast to the PWD responses of SAP SNL rats, whose ipsilateral paws retained hypersensitivities to all of the applied stimuli throughout the testing period, being equally elevated on PO day 2 and PO day 14. The time-related

reversal of heightened sensitivities to tactile stimuli in the derm-SAP SNL rats probably represents a key difference between mechanisms that initiate neuropathic pain and mechanisms that maintain neuropathic pain, as speculated previously (Porreca et al., 2001, Burgess, 2002, Vera-Portocarrero et al., 2006). Specifically, given the similar ipsilateral PWD frequencies of the two (differently injected) groups of SNL rats on PO day 2, it seems unlikely that descending facilitatory neurones are required at this early stage to uphold hypersensitive responses. However, the progressive attenuation of ipsilateral PWD responses to the range of stimuli after PO day 2 in the derm-SAP SNL rats suggests a dependence on the descending facilitatory system for behavioural support of mechanical hypersensitivities at this later stage (thus when it is not present, behavioural responses decline). In essence, ablating RVM MOR cells has a protective effect against SNL-induced nociception at later time-points, which implicates the descending facilitatory system in the maintenance, but not the initiation, of neuropathic pain.

This dichotomy has been explored before, since DLF lesions and intra-RVM lignocaine in SNL animals have been shown to affect behavioural indices of pain at late PO days only (Porreca et al., 2001, Burgess, 2002). Fos, the protein that is encoded by the immediate early gene *c-fos*, which is a marker of neuronal excitability and spinal sensitisation when present in dorsal horn neurones (Hunt et al., 1987), has an expression profile that temporally matches the development of behavioural hypersensitivities in SNL animals (Vera-Portocarrero et al., 2006). Fos expression can be blocked by antagonising spinal NMDA receptors, which themselves are critical for central sensitisation (Chapman et al., 1995). Moreover, spinal Fos expression in SNL animals can be blocked by pre-lesioning descending facilitatory neurones, yet this only occurs after PO day 3 which strengthens the concept that once initiated, maintenance of nerve injury-induced central sensitisation in the dorsal horn requires descending facilitatory input (Vera-Portocarrero et al., 2006). Interestingly, like the decline in behavioural hypersensitivities after PO day 3 in derm-SAP SNL rats, the decrease in spinal Fos expression occurred gradually, with partial expression still apparent at PO day 5 (which correlates with the slightly elevated PWD responses at this time-point). It therefore seems that the so-called 'maintenance mechanisms' steadily take effect, overlapping with the initiation mechanisms on several days as they become increasingly responsible for the pain phenotype. The separation between

'initiation' and 'maintenance' mechanisms may therefore be more heuristic than literal, with each underlying process having an increased or decreased time-related order of relevance. Thus just because descending facilitatory pathways are given 'maintenance' status, it does not necessarily mean their contribution is negligible during the early phases of nerve injury, just that other mechanisms may be more important.

One of these other mechanisms is spontaneous afferent drive from the periphery; within 24 hours of nerve injury there is an estimated four-to-six fold increase in ectopic firing from injured or adjacent nerves (Liu et al., 2000), which correlates with the early onset of behavioural hypersensitivities (Sun et al., 2005). However, this discharge, which peaks within several days, tapers off over the course of a week (Han et al., 2000) and becomes insufficient to maintain neuropathic behaviours that can persist beyond 7 days, and possibly up to four months (Kim and Chung, 1992). Interestingly, ectopic activities are significantly diminished by PO day 5, which is the approximate time that descending facilitatory neurones acquire greater pathophysiological status. Hence rather than descending pathways 'stepping in' to rescue waning excitability and bolster spinal facilitation, it could reasonably be the case that their role is unmasked at this time point. On a similar note, even though aberrant neuronal activity in the periphery is said to underlie the genesis of neuropathic pain, spontaneous activity of WDR neurones in the dorsal horn, with mean discharge rates between 2.5-3.5 Hz, can still be recorded *in vivo* more than 14 days after SNL surgery (Chapman et al., 1998b, Chu et al., 2004, Suzuki and Dickenson, 2006). This ongoing activity, although significantly reduced with respect to activity at earlier time points, may still be important for maintaining the neuropathic phenotype and should not therefore be consigned solely as an 'initiation' mechanism (Sheen and Chung, 1993, Yoon et al., 1996).

The interplay between peripheral and supraspinal mechanisms in neuropathic pain has been demonstrated experimentally, since at later time-points in SNL rats, enhanced evoked excitatory transmitter release from peripheral nerves has been shown to require descending facilitatory input from the RVM (Gardell et al., 2003); Even though this study's main conclusion pertains to the important role of the descending facilitatory system in maintaining neuropathic pain, it also demonstrates

the continued contribution of the peripheral nervous system to this state. The required convergence of different pathophysiological mechanisms probably explains why the application of mustard oil to a rat's leg (which increases the firing of RVM On cells) produces hyperalgesia in the treated limb only (Kincaid et al., 2005). Given the global influence of RVM neurones and their bilateral projections to several spinal cord segments (Huisman et al., 1981, Fields et al., 1995b), it is initially surprising that the hyperalgesia is so confined. One possible explanation for this restriction is that activation of On cells may be insufficient to produce behaviourally measurable hyperalgesia in the absence of peripheral drive (just as peripheral drive alone may be insufficient to produce behavioural hypersensitivities, as demonstrated in my study). Alternatively, descending inhibitory neurones may be recruited in parallel with descending facilitatory neurones to suppress excitability in circuits serving other regions of the body.

This hypothesised heterotopic inhibition is akin to diffuse noxious inhibitory control whereby noxious stimuli activate inhibitory controls to sharpen the contrast between the stimulus zone and adjacent areas (having a net enhancing effect on the perceived intensity of the painful stimulus, and an analgesic effect in other areas) (Dickenson et al., 1981). Descending facilitations from the RVM can however induce hyperalgesia in both sides of the body after unilateral muscle insult (e.g. injecting acidic saline into the gastrocnemius muscle of the left hindlimb) (Tillu et al., 2007). This animal model is thought to replicate many features of human chronic widespread muscle pain (CWMP) (Gran, 2003), a condition characterised by alterations in descending modulatory controls, and in particular a loss of descending inhibitions (Kosek and Hansson, 1997). Hence, according to the theory above, this loss could explain the bilateral nature of the hyperalgesia. In the CWMP model, descending facilitations are thought to be critical for both the initiation and maintenance phases of pain, since local anaesthetic injections in the RVM at given time-points prevented the development of behavioural hypersensitivities and reversed established pain behaviours (Tillu et al., 2007). The idea that different pain models elicit different patterns of descending controls is strengthened by observations that descending facilitations predominate in the early stages of some tissue injuries but eventually give way to a descending inhibitory drive (Guan et al., 2002, Terayama et al., 2002). Furthermore, differences in the quality and pattern of descending controls may relate

to the location of the injury, since formalin injections in the muscle increase *c-fos* expression primarily in the ventrolateral PAG, whilst formalin injections in the skin induce *c-fos* expression primarily in the lateral PAG (Keay and Bandler, 1993). Some of these non-localised changes in pain responsivity through descending influences could relate to diffuse pains such as fibromyalgia.

Like neuropathic pain mechanisms, the detailed time-course of analgesic drugs is often difficult to resolve. For example, brief infusion of systemic lignocaine in SNL rats shows 3 distinct phases of relief regardless of its delivery at PO day 2 or 7 (Araujo et al., 2003). These phases include i) acute reduction in behavioural hypersensitivities during lignocaine infusion, ii) transient reduction within 6 hours after infusion, and iii) sustained reduction that develops slowly several days after infusion and is still evident 3 weeks later (despite the relatively fast plasma clearance of lignocaine). The most obvious explanation for these effects is that lignocaine reduces ectopic activities from injured and adjacent nerves (Chabal et al., 1989, Abdi et al., 1998), which therefore reduces their supraspinal priming potential. However other agents that block VGSCs and cause equable inhibition of neuronal activity, do not reduce behavioural hypersensitivities over several days, which indicates that the acute and sustained effects of lignocaine are not obligatorily coupled. This is emphasised further by the separation between the three relief phases listed above; the long-term effects of lignocaine only occurred after a complete return to pre-infusion hypersensitive states. The lack of smooth transition between the acute and chronic effects of lignocaine therefore suggests that mechanistic differences separate its acute and chronic analgesic effects.

Regardless of when exactly descending facilitations start contributing to the pain state, it is most likely the case that a barrage of afferent inputs from the periphery primes central mechanisms to elicit neuroplastic changes that result in increased activation of descending facilitatory pathways. Accordingly, clinical reviews of chronic pain after surgery conclude that untreated acute post-operative pain is the greatest predictive factor for the development of chronic pain, which not only validates the 'trigger theory' but the principle of pre-emptive analgesia too (Perkins and Kehlet, 2000). There are however clinical trials where pre-emptive analgesia failed to have an effect (Kelly et al., 2001), yet it is thought that treatments therein

were either not long enough, or did not fall within a critical 4-5 day window of opportunity essential for assuaging the post-operative nociceptive barrage.

There is currently considerable interest in the pre-operative analgesic potential of GBP and PGB since eight placebo-controlled trials have reported significantly lower pain scores after surgery following pre-emptive GBP dosing (Seib and Paul, 2006). However, regardless of the small number of subjects used in each of these trials, the results may be secondary to an anxiolytic effect, since in a separate trial, GBP given two hours before surgery significantly reduced post-operative VAS anxiety scores (Menigaux et al., 2005). Hence, whilst the pre-operative effects of GBP or PGB may be potentially valid, they may not reflect analgesic effects *per se*. It would be difficult to reconcile a true pre-operative analgesic effect of GBP with current knowledge of its action, since the large consensus is that in both animals and humans, this agent only confers dose-dependent efficacy in the presence of sensitisation, which may take several days to reach full effect (Jones and Sorkin, 1998, Iannetti et al., 2005, Suzuki et al., 2005) (N.B. although there may be novel mechanisms that underlie GBP's hypothesised pre-operative actions that have yet to be elucidated). It is telling therefore that in my studies, PGB failed to inhibit dorsal horn neuronal responses to the range of stimuli used in SNL rats that had been pre-injected with derm-SAP, or in SAP-SNL rats during the early post-operative period.

Given the delayed onset of PGB's efficacy demonstrated in these experiments (which could be circumvented by stimulating spinal $5HT_3$ receptors), it would be interesting to investigate the time-course of gabapentinoids in the clinic, and specifically to see whether they have any efficacy in the immediate aftermath of nerve injury. There is not much evidence of this at present, possibly because it takes patients several weeks, if not months and years to report pain after damage to the nervous system. However, one trial has reported that a single dose of gabapentin in patients infected with VZV reduces the acute pain and allodynia associated with PHN, which hints at a carry-over effect and suggests that GBP may prevent development of painful neuropathies by reducing pain and central sensitisation during the acute infection phase (which contradicts the possibility of late-onset action) (Berry and Petersen, 2005).

As expected on post-operative day 14, neurones in SAP-injected SNL rats were susceptible to PGB's inhibitory effects, and the large majority of responses to electrical, mechanical and thermal stimuli were significantly reduced after the systemic injection of 10mg/kg PGB. Thus it seems that ablation of medullary MOR cells in the derm-SAP rats not only impacts behavioural hypersensitivities and coding properties of neurones after SNL (since neuronal responses to C-fibres, input, vF 26g and 60g were all lower in these animals than in SAP SNL animals), but it also affects responsiveness to PGB. Interestingly, two weeks after SNL surgery, qPCR against $\alpha_2\delta_1$ and $\alpha_2\delta_2$ mRNA from injured and uninjured DRGs showed that between 'normal' SNL rats (i.e. rats that had not received any RVM injections) and derm-SAP SNL rats, mRNA levels were consistent for each equivalent DRG that was extracted. Hence, in both groups of rats, there was significant upregulation (relative to contralateral levels) of $\alpha_2\delta_1$ mRNA in the ipsilateral L5/6 (injured) DRGs and significant downregulation of $\alpha_2\delta_2$ mRNA, whilst ipsilateral L4 (uninjured) DRGs had unaltered levels of $\alpha_2\delta_1$ and $\alpha_2\delta_2$ mRNA. Thus in rats that had received intra-RVM injections of derm-SAP, lesioning injuries to L5 and L6 spinal nerves produced the same alterations in DRG $\alpha_2\delta$ subunit levels that were seen in normal SNL rats, which means that PGB's lack of effect in the former group was not a consequence of reduced $\alpha_2\delta$ protein and hence peripheral binding sites.

In neuropathic states there is enhanced $5HT_3$ receptor-mediated control of deep dorsal horn neurones, as evidenced by ondansetron's increased inhibitory effect in SNL rats relative to normal rats (Suzuki et al., 2004). A marked increase in 5HT-containing neurones in the spinal cord is probably relevant for the behavioural and neuronal hypersensitivities after nerve injury, and thus the enhanced effects of spinal ondansetron (Oatway et al., 2004). Interrupting descending facilitatory pathways by ablating RVM MOR-expressing neurones seems to directly influence PGB's efficacy, because when effects of this drive were mimicked *in vivo* in derm-SAP SNL rats by spinal application of the $5HT_3$ receptor agonist 2-me5HT in the presence of PGB, neuronal responses became inhibited to the range of electrical, mechanical and thermal stimuli used, and this occurred to approximately the same extent that responses were inhibited by PGB alone in SAP SNL rats (~40% reduction of pre-drug control responses for all measures, although in SAP SNL rats only, PGB reduced responses to noxious thermal stimuli by ~60%). This induced inhibitory effect in

derm-SAP SNL rats was not due to 2-meHT's actions *per se*, since on its own it did not significantly alter neuronal responses. Given that this agonist activates facilitatory $5HT_3$ receptors in the spinal cord, it is perhaps surprising that 2-me5HT did not *enhance* dorsal horn neuronal responses in derm-SAP SNL rats when applied singly, and essentially restore responses to pre-drug levels seen in SAP SNL rats (seeing as the reduced baseline coding in derm-SAP rats, nerve injured or otherwise, is probably a consequence of reduced pre-synaptic serotonergic inputs). There was a trend towards increased responses to mechanical stimuli after the application of 2-me5HT, particularly at the higher threshold end, yet these did not merit statistical significance. It could be the case that alterations in spinal cord circuitry confer marked changes in spinal serotonin function, unmasking $5HT_3$'s 'inhibitory' actions (as described in Chapter 6) to offset the otherwise facilitatory effects of 2-me5HT.

These results support a permissive role of spinal $5HT_3$ receptors that has been described previously (Suzuki et al., 2005). This role is fully functioning in SAP SNL rats, which is why PGB effectively inhibited neuronal responses, yet it was only revealed in derm-SAP SNL rats by manipulating spinal $5HT_3$ receptors. Essentially, spinal $5HT_3$ receptors need to be active for PGB to have inhibitory effects in the spinal cord and the RVM has to be intact. This requirement, and not alterations in peripheral $\alpha_2\delta$ subunits, probably underlies the state-dependent actions of GBP and PGB. The coincident presynaptic locations of VGCCs and $5HT_3$ receptors on the terminals of small diameter primary afferent neurones permits an interaction between their respective activities, and it is this interaction that GBP and PGB are effective against. The behavioural results from derm-SAP SNL rats in this study suggest that descending facilitatory inputs do not strongly influence the pain phenotype at early post-operative time points, which intimates a reduced role of 5HT (acting at $5HT_3$ receptors) at this stage. Given this supposition, and the hypothesised interaction between $5HT_3$ receptors and VGCCs that is essential for PGB's inhibitory actions, PGB should lack efficacy in SAP SNL rats (as well as derm-SAP SNL rats) at early PO time-points. Indeed, in both derm-SAP and SAP rats undergoing electrophysiological testing 2-4 days after SNL surgery, systemic injection of 10mg/kg PGB failed to inhibit neuronal responses to any of the applied stimuli. The intrathecal addition of 2-me5HT reduced neuronal responses to vF 26g and 60g in both groups of rats.

These results confirm the requirements for an intact spino-bulbo-spinal loop, and therefore descending facilitatory controls, for the inhibitory actions of PGB. Thus PGB fails to inhibit neuronal responses when the loop is interrupted (by ablating medullary MOR expressing cells), or when descending facilitatory controls have little influence (i.e. during the early days after SNL surgery). Its inhibitory effects can however be rescued and restored by manipulating spinal $5HT_3$ receptors. These novel requirements, which probably determine the state-dependent actions of PGB and GBP, are specific for the actions of this class of analgesic drugs since intrathecal morphine effectively inhibited neuronal responses in both derm-SAP and SAP SNL rats in a dose-dependent and naloxone-reversible fashion (which therefore implies actions at spinal μ-opioid receptors).

In the dorsal horn of the spinal cord μ-opioid receptors are present on both pre- and postsynaptic membranes which enables them to respectively attenuate glutamatergic synaptic inputs into the spinal cord and reduce responses of dorsal horn neurones (Dickenson and Sullivan, 1986, Fleetwood-Walker et al., 1988, Schneider et al., 1998). There is prominent MOR expression in the terminals of primary afferent fibres entering laminae I, II and V of the dorsal horn at all segmental levels (Besse et al., 1990), and in particular MOR-ir occurs in DRG neurones that do not stain positive for RT97, a neurofilament marker of large, myelinated primary afferents (Arvidsson et al., 1995). This suggests that μ-opioid receptors localise in small-to-medium diameter neurones that give rise to unmyelinated primary afferent fibres, a conclusion that is upheld by morphine's lack of effect on Aβ-fibre evoked responses (Suzuki et al., 1999). Following their synthesis in the cell body, presynaptic opioid receptors are targeted to membrane areas subjacent to the site of transmitter release, and so their activation, which is coupled to intracellular G_i protein stimulation, results in reduced exocytosis of excitatory transmitters into the synaptic cleft.

Despite early clinical reports that neuropathic pain is an 'opioid-resistant' state (Arner and Meyerson, 1988), morphine is efficacious in rat models of neuropathy, both electrophysiologically when spinally delivered (Suzuki et al., 1999, Rashid et al., 2004, Chen et al., 2006), and behaviourally when systemically injected (Kontinen et al., 1998), and more recent clinical studies concur with these preclinical conclusions (Rowbotham et al., 1998). Spinal morphine's potentially reduced inhibitory effect

following nerve injury could be the consequence of down-regulated MOR expression in discrete dorsal horn areas ipsilateral to the side of nerve injury (Porreca et al., 1998). Alternatively, or in addition, morphine's slightly reduced efficacy in the nerve-injured state may be causally related to reduced GABAergic tone in the dorsal horn following nerve injury (Ibuki et al., 1997, Torsney and MacDermott, 2006); there is extensive co-labelling of GABA and MOR in lamina II (Kalyuzhny et al., 2000) and MOR expressing GABAergic neurones therein are thought to partly account for morphine's spinal inhibitory actions, particularly with respect to deep dorsal horn neurones (Magnuson and Dickenson, 1991). This incidentally may explain the paradoxical low-dose *facilitation* of neuronal responses to some peripheral stimuli in my study following spinal topical application of 0.25μg morphine.

A more salient feature of my morphine study however relates to the reduced inhibitory capacity of low-dose spinal morphine in derm-SAP SNL rats relative to SAP SNL rats; at a higher dose (1μg), morphine inhibited a range of neuronal responses to a similar degree in both groups of SNL rats, yet 0.25μg morphine had a comparatively stronger inhibitory effect in SAP SNL rats. It has recently been reported that ablation of superficial NK1 receptor expressing neurones by lumbar injections of SP-SAP affects $GABA_A$-receptor mediated activity in the spinal cord of normal rats (Rahman et al., 2007), which may be due to a loss of GABAergic inhibition in these animals (yet despite this purported inhibitory loss, there is a net reduction in spinal cord excitability in SP-SAP rats which indicates the facilitatory strength of an intact spino-bulbo-spinal loop in normal animals (Suzuki et al., 2002b)). It is suggested that the link between these projection neurones and the consequences of blocking spinal GABAergic circuitry may reside in intrinsic spinal synaptic contacts, or through activation of descending controls. Hence, when these descending controls are interrupted, for example by ablating RVM MOR cells, spinal GABAergic tone may be altered (above and beyond the level of alteration incurred by SNL surgery). This is a potential explanation for the reduced inhibitory capacity of low-dose morphine in the derm-SAP SNL rat. It is unlikely that this reduced efficacy was a direct consequence of RVM MOR cell loss since a higher dose of morphine could inhibit responses perfectly well. Thus it seems that state-dependency is the preserve of pregabalin's actions.

In summary, results from this study demonstrate the importance of supraspinal structures and in particular descending facilitatory input from the RVM in maintaining a sensitised state following damage to the nervous system. Furthermore, the results indicate an interaction between central sensitisation and PGB's efficacy that depends on an operant descending serotonergic system. Serotonin has a well-described role in setting mood and emotions, and so together with the fact that midbrain areas involved in the processing of fear, stress and anxiety are contacted by spino-bulbo-spinal circuits, and activity in the ACC directly impacts descending modulatory structures (Calejesan et al., 2000), this system may represent an anatomical inter-dependence between the sensory and emotional components of pain, and thus the neural substrate through which emotions can influence pain, and reciprocally a pathway through which pain can influence 'state of mind'. This not only provides a theoretical basis for the co-morbid presentations of depression, anxiety and sleep-disorders (for example) with chronic pain, but also potentially explains why there are variable responses to treatments such as gabapentin and pregabalin in homogenous pain groups (Sindrup and Jensen, 1999). Indeed, despite consistent efficacy in most animal models of neuropathic pain, there is an ill-defined link between the presence of nervous system lesions or abnormal sensory phenomena, and responsiveness to GBP (and therefore probably PGB too) in the clinic, with the former not accurately predicting the latter (Rasmussen et al., 2004). Given that GBP and PGB's efficacy seems to relate to activity in the $5HT_3$ receptor-mediated spino-bulbo-spinal loop, psychology and level of vigilance may confound treatment outcome, which therefore means that a combination of approaches, pharmacological and otherwise, may be necessary to align the pathophysiology back to a normal state.

8. FINAL DISCUSSION

Sensory information arriving in the dorsal horn of the spinal cord is subject to extensive processing and filtering before it is transferred to supraspinal structures for interpretation. An adaptive relationship is therefore said to exist between injury and pain such that stimulus intensity and perceived pain do not always correlate. Instead, at any given time and for any given stimulus, a range of internal and external factors can influence the fidelity of sensory discrimination and the coupling between input and output, thus allowing the responsiveness of the sensory system to adhere to the conflicting demands of the organism.

In this thesis, I have described a pathway that descends from the RVM to the dorsal horn of the spinal cord which impacts upon wide-dynamic-range neurones in deep laminae. This pathway is CCK-sensitive and uses 5HT as a major neurotransmitter. My results have shown that the balance between RVM descending facilitatory and inhibitory neurones in this pathways in the normal state weights in favour of gain control, particularly with respect to higher threshold stimuli. Hence, descending pathways can preferentially modify neuronal responses to noxious versus innocuous stimuli, which ensures the detection of noxious inputs and promotes the useful biological function of pain as a signal of impending or actual tissue damage. The balance between supraspinal inhibition and facilitation can however be reversibly and transiently altered so that pain signals are enhanced or suppressed. With respect to the latter, descending inhibitory pathways can be activated and endogenous analgesia recruited so that nociception and protective reflex responses to noxious stimuli are temporarily suppressed in situations of threat and danger, yet when the threat is removed and safety signals resume, nociceptive sensitivity is normalised by hyper-activity in the descending facilitatory pathway (Wiertelak et al., 1992, Watkins et al., 1994). In this aftermath, and in situations of pain that are not associated with threatening situations, descending facilitatory drive may increase to encourage protective and recuperative behaviours, therefore limiting further tissue damage in accordance with the long-term survival needs of the organism.

The sensory system may however become maladaptive and durably altered so that pain outlasts its cause and biological usefulness. After damage to the nervous

system for example, aberrant neuronal activity can increase the descending facilitatory drive to maintain the pain state, yet after the injury is healed, excessive facilitatory neurones may fail to disengage which means that they continue to enhance the transmission of sensory input. Since one mechanism by which they achieve this is through enhancing the evoked transmitter release from peripheral nerves (Gardell et al., 2003), the feed-forward compensatory circuit between the periphery, spinal cord and supraspinal structures becomes self-sustaining and gives rise to chronic pain. Cells expressing μ-opioid receptors in the RVM are particularly relevant to this pathophysiology (Burgess, 2002). As I have shown in this thesis, medullary MOR expressing cells equip the spinal cord with full sensory coding capacity in the normal state, whilst in the nerve-injured state they additionally maintain central sensitisation and behavioural manifestations of pain.

Many spinally projecting serotonergic neurones in the RVM express μ-opioid receptors (Kalyuzhny et al., 1996), and so this facilitation and enhancement of responses is mediated, at least partly, by serotonin acting on spinal facilitatory $5HT_3$ receptors. It should be mentioned however that MOR expression is not a defining characteristic of serotonergic drive, nor is it the exclusive mark of descending facilitatory cells (Marinelli et al., 2002), instead many intrinsic RVM neurones express μ-opioid receptors that may serve to disinhibit Off cells during morphine analgesia. Nevertheless, the consequences of targeted ablation of MOR-expressing cells in the RVM by dermorphin-saporin include reduced expression of the pain phenotype following SNL surgery, and lost efficacy of systemic PGB, which suggests that the influence of MOR agonists on the facilitatory system is greater than its influence on the inhibitory system. Since RVM facilitatory cells are integral to the spino-bulbo-spinal loop, and the spino-bulbo-spinal loop reaches brain areas that co-ordinate the sensory and affective components of pain, activity therein may influence not only painful outcome but responsiveness to treatment too. Thus, individual psychological contributors and cognitive set may explain why the same nerve injury presents in different people in different ways (or has different effects in the same person at different times according to the variable and intermittent nature of neuropathic pain), as well as the variable responses of patients to analgesics (Sindrup and Jensen, 1999). Furthermore, abnormal activity in this loop may potentially

explain some diffuse pain states that lack simple causal labels, such as CRPS and fibromyalgia.

The definition of fibromyalgia is based on a set of specific symptoms that must include wide-spread pain in all quadrants of the body for a minimum of 3 months, tenderness at least 11 of 18 specific points (Wolfe et al., 1990) and increased sensitivity to pressure, cold, heat and electrical stimulation (Gracely et al., 2003). These signs and symptoms are difficult to explain by peripheral mechanisms since there is no obvious peripheral neuronal pathology, and histological examination of muscle biopsies from affected patients show no obvious tissue abnormalities (Schneider et al., 1998). Thus, by deduction it seems that the pathogenesis of this condition is centrally-based, and several features of fibromyalgia's presentation and treatment support this idea. Firstly, the altered pattern of sensory processing that manifests in wide-spread allodynia is symmetrically distributed to the rostrocaudal axis of the body suggesting abnormal central activity. Secondly, relative to healthy controls, patients with fibromyalgia have a greater level of temporal summation following repetitive stimulation with a thermal stimulus, which indicates NMDA-receptor mediated wind-up in the dorsal horn (Staud et al., 2004). This central sensitisation hypothesis is backed by the capacity of ketamine, a NMDA-receptor antagonist, to reduce areas of referred pain (Graven-Nielsen et al., 2000). Finally, central disruptions are indicated by elevated levels of Substance P in the CSF and altered 5HT metabolism in fibromyalgia patients (Vaeroy et al., 1988). This latter point may explain the rationale for using TCAs, and in particular amitriptyline to relieve painful symptoms in affected patients (Clauw and Crofford, 2003). Moreover, the efficacy of these anti-depressants could more generally reflect an underlying dysfunction in the descending pain modulatory system that needs correction. Thus, whilst the periphery remains apparently normal, exclusive central mechanisms that may either involve enhanced descending facailitations and/or impaired descending inhibitions may be responsible for the pathogenesis and presentation of fibromylagia, which, given the links between the sensory and affective components of pain and the descending modulatory system, may also help explain the hypothesised psychosomatic and psychogenic basis of fibromayalgia (Rubin, 2005).

Abnormal supraspinal activity may additionally explain opioid-induced hyperalgesia and the related phenomenom of paradoxical pain. Many chronic pain states require increasing doses of opioids to maintain adequate pain relief, which, in addition to disease progression, is thought to be the consequence of opioid tolerance (defined as a decrease in efficacy after previous exposure to the same, or similar, drugs (Way et al., 1969)). Moreover, many clinical reports have noted that opioids administered through different routes can unexpectedly produce hyperalgesia and allodynia that often manifest in locationally and qualitatively distinct manners to the original pain complaint, particularly during rapid dose escalation (Blum et al., 2003, Mercadante et al., 2003). Related observations have been validated pre-clinically in animal models (Collin and Cesselin, 1991, King et al., 2007), with early mechanistic interpretations citing cellular and peripheral adaptions. However, it is increasingly thought that opioid-induced hyperalgesia may be secondary to neuroplastic changes at supraspinal sites, with up-regulated CCK activity promoting descending facilitations from the RVM to offset spinal opioid inhibitions (Gardell et al., 2006). Consequently, physical disruptions of the DLF pathway can block the hyperaesthetic behaviours associated with sustained opioid delivery (Gardell et al., 2006) in a similar way that CCK receptor antagonists can (Kellstein et al., 1991). Sustained triggering of descending facilitatory influences from the RVM may act in concert with spinal mechanisms such as wind-up to enhance central sensitisation, giving rise to the diffuse pains associated with fibromyalgia and prolonged opioid use, which in each circumstance can be treated with antagonists acting at spinal NMDA or $5HT_3$ receptors (Lutfy et al., 1996, Farber et al., 2000, Graven-Nielsen et al., 2000).

In agreement with animal studies and in parallel with my pregabalin data, *f*MRI studies in human subjects have shown that the efficacy of GBP relates to activity in spinal-brainstem circuits (Iannetti et al., 2005). Specifically, increased activity in the brainstem after mechanical stimulation of sensitised skin (which probably reflects the engagement of descending facilitatory influences during central sensitisation) was selectively reduced by GBP in line with its anti-hyperalgesic effects. Interestingly, these studies also showed that activities in discrete brain regions that did *not* include the brainstem, thalamus and ACC, were consistently reduced by noxious stimulation in a GBP-reversible fashion (i.e. GBP inhibited brain deactivations). Stimulus-evoked decreases in *f*MRI signals reflect reductions in

neuronal activity that correspond to a default mode of brain activity (Raichle et al., 2001, Shmuel et al., 2002, Stefanovic et al., 2004), and so in this study, brain efforts were essentially concentrated into areas involved in the processing of the painful stimulus. Importantly, these deactivations, which could be induced in the normal state by nociceptive stimulation, were significantly increased in the sensitised state and covered a greater proportion of the brain, suggesting a "greater shift or processing resources towards a stronger and attentionally demanding stimulus" (Iannetti et al., 2005). The subjugation of other supraspinal activities in favour of attending to the pain can however enhance its experience (Tracey et al., 2002).

The *f*MRI study looking at attentional modulation of pain was one of the first imaging studies of nociceptive processing in human brainstems (Tracey et al., 2002). Prior to this, inherent difficulties in imaging subcortical areas meant that human correlative evidence of the brainstem's involvement in nociception was lacking, yet improved technology in recent years has meant that whole-brain high-field *f*MRI is now possible. This has enabled human supraspinal areas that contribute to pain perception and its modulation to be determined, thus confirming the relevance of nociceptive nuclei identified in animal studies.

Nociceptive stimulation of the lower rectum has been shown to activate structures in midbrain and brainstem areas that are consistent with the PAG, NC, ventral tegmental area, nucleus coeruleus and parabrachial area (Dunckley et al., 2005). Furthermore, a separate study has shown that brainstem activations during painful stimulation are significantly localised in two distinct areas of the midbrain reticular formation in regions consistent with the location of the nucleus cuneiformis and the PAG (Zambreanu et al., 2005), areas that collectively form the major source of input to the RVM (Behbehani and Zemlan, 1986). Drawing additional parallels with animal data, these areas have been shown to have enhanced activity during central sensitisation (Zambreanu et al., 2005). Similar conclusions have been reached in experiments looking at the patterned activity evoked by cold allodynia and cold pain in human subjects (Seifert and Maihofner, 2007); specifically, cold allodynia was shown to activate networks similar to those activated by cold pain (of equal intensity), yet additionally recruited areas within the dorsolateral pons that were consistent with the parabrachial area, verifying the specialised role of this supraspinal site in central

sensitisation. Furthermore, comparison analyses of map activity in the spinal trigeminal nucleus and brainstem during dynamic mechanical allodynia highlighted neuronal changes in many brainstem nuclei relative to control stimulation, with increased activity in the ipsilateral caudal dorsal medulla/upper cervical cord in an area consistent with the location of the RVM being particularly prevalent during dynamic mechanical stimulation of the area of primary hyperalgesia (Mainero et al., 2007).

Together with recent diffusion tractography techniques that have confirmed anatomical pathways that mediate top-down control of nociceptive processing in humans (Hadjipavlou et al., 2006), the imaging data provide a clear picture of the brainstem's representation of pain and central sensitisation in humans, and consequently paves a route from bench to clinic by consenting safe transfer of knowledge acquired in animal studies of chronic pain to humans.

In addition to attentional modulation of pain, anticipation can sufficiently raise the level of anxiety to increase the perception of pain in a feed-forward, reinforcing manner (Hill et al., 1952). The reported anxiolytic and pre-operative analgesic actions of GBP may therefore be tightly coupled to its ability to reduce brain deactivations (to consequently reduce attention to the impending stimulus), as well as its ability to reduce brainstem *activations*. It is noteworthy that amongst the potential therapeutic applications of GBP that are currently being trialled in the clinic[9], there is a suggestion that this agent might affect mood and emotional processing, an idea that is in keeping with the current hypothesis of the close anatomical link between the sensory and affective components of pain.

As well as GBP's hypothesised effect on emotional processing, there are also anecdotal reports of its potential use in the treatment of chemotherapy-induced nausea and vomiting (CINV) (Guttuso et al., 2003, Ho, 2006). It has been suggested that these effects may reflect reduced opioid-related side-effects (since fentanyl requirements are lower in GBP-treated patients), yet GBP's efficacy has also been described with respect to the sickness associated with cholecystectomy and pregnancy[9] (Pandey et al., 2006). This is interesting given the involvement of the

[9] www.clinicaltrials.gov

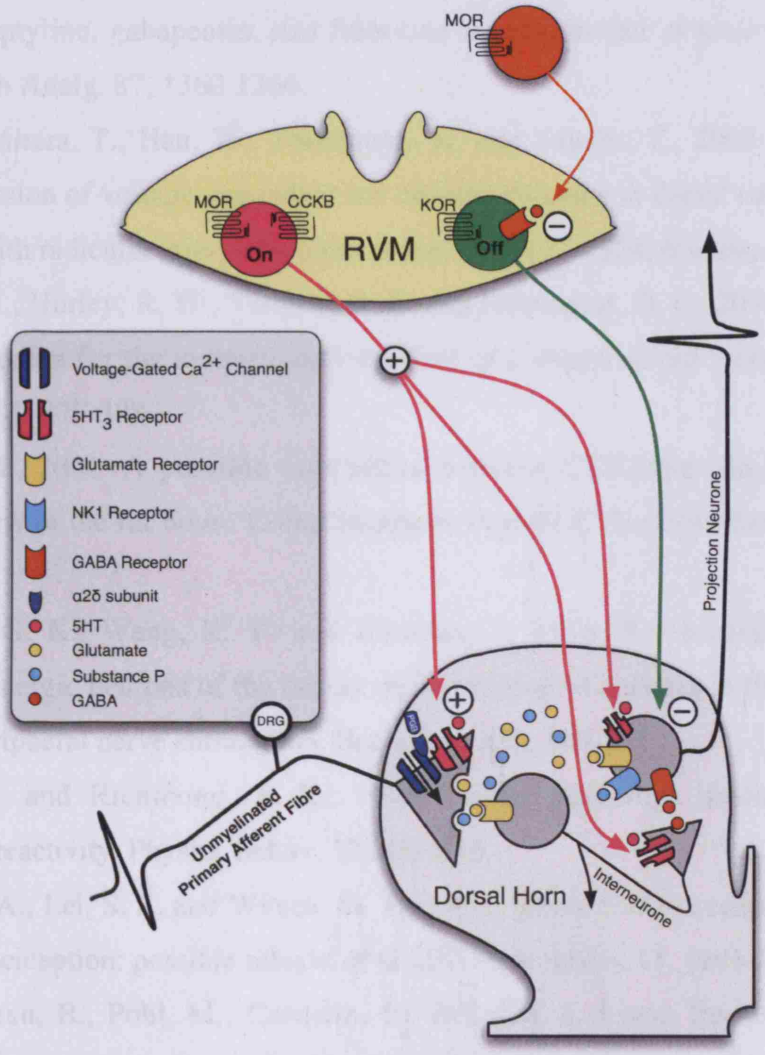
serotonergic system and $5HT_3$ receptors in emesis (Reed, 1992). It could hypothetically be the case that in addition to their spinal anti-hyperalgesic actions, GBP and $5HT_3$ receptors interact in the vomiting centre in the medulla to affect nausea. Furthermore, Arepitant, an agent that has selective high affinity antagonist action at NK1 receptors, has recently been FDA approved for the treatment of CINV (Diemunsch et al., 2007). This emetic involvement of the serotonergic system, GBP and neurones expressing NK1 receptors is reminiscent of their interplay in neuropathic pain states (Suzuki et al., 2005). Parallels can be drawn between nausea and nociception since both are unpleasant sensations that subserve biological warning functions, both are multifactorial in origin and can be triggered by excessive activation of chemoreceptors and mechanoreceptors, and both are common post-operative complications that can be amplified by anticipation (Watson et al., 1992). Together with the transferable ability of the CCK and 5HT systems to modulate nociception and GI function (described in Chapter 5), these observations imply consistencies in the processing of various external inputs (such as pain, fear, GI distention and the triggers for nausea and vomiting), pointing towards a unified theory of positive symptoms in the event of pathophysiology.

This paradigm links neuropathic pain with tinnitus, a condition that is characterised by the perception of noise in the ear in the absence of corresponding external sound(s). Like neuropathic pain, tinnitus is a subjective experience that is caused by hyperexcitability and spontaneous activity in the nervous system. The most common form of chronic tinnitus, 'acquired centralised tinnitus' (ACT), is initiated in the periphery by a sensitising signal and is maintained by neuroplastic changes and facilitatory processes in the auditory system (akin to plastic changes and facilitatory domination in the brainstem following damage to peripheral nerves), thus resulting in the maladaptive processing of sound (Zenner et al., 2006). The mechanistic similarities between neuropathic pain and ACT provide a theoretical basis for the use of gabapentin in the treatment of the latter (Bauer and Brozoski, 2006). It is remarkable that despite the very different clinical characteristics of tinnitus and neuropathic pain, their associated symptoms arise from lesions to the nervous system, and in each case positive symptoms can co-exist with sensory deficits. Increased understanding of the multiple compensatory processes that underlie one or the other of these pathophysiologies could lead to identification of novel therapeutic targets at

various sites within the nervous system, lending hope to the future development of new analgesics and tinnitus-treating drugs.

In conclusion, increased knowledge of the descending facilitatory system in chronic pain states calls for a fresh approach to pain relief, with new therapies based on new understanding. The causes of neuropathic pain are diverse and can range from metabolic disturbances to viral infections to crushing injuries, yet symptoms are fairly consistent in the patient population (Hansson, 2002). Drugs that target these symptoms presently have the monopoly on pain control, yet given the differences in underlying processes and the occasionally refractory nature of different pain states, the concept of mechanisms-based treatments needs to be appraised, and in the absence of a pharmacological elixir, the cumulative effects of combined therapies needs to be explored. Finally, it is hoped that the relationship between signs and symptoms and treatment outcomes will be accurately mapped and understood, so that pain control is shifted from the margins of medicine towards the forefront of GP's attention.

Figure 8.1 *Summary diagram showing the anatomical link between the periphery, spinal cord and supraspinal areas.*



The RVM gives rise to facilitatory On cells and inhibitory Off cells that can modulate the processing of sensory information in the dorsal horn of the spinal cord. RVM Off cells receive an extrinsic GABAergic input that is sensitive to mu-opioid agonists (hence Off cells are disinhibited by MOR agonists) and their firing inhibits spinal cord activity. On cells, which can be directly inhibited by mu-opioid receptor agonists, and excited by CCK_B receptor agonists, excite the spinal cord by releasing 5HT onto $5HT_3$ receptors that are located on the presynaptic terminals of primary afferent fibres entering the dorsal horn of the spinal cord, and also via actions on post-synaptic projection neurones. Supraspinal 5HT acting at spinal $5HT_3$ receptors may paradoxically inhibit spinal cord activity by activating inhibitory interneurones within the dorsal horn. Presynaptically, $5HT_3$ receptors colocalise with voltage-gated Ca^{2+} channels, an interaction that may be permissive for the state- and time-dependent inhibitory actions of pregabalin.

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